application of bacterial mutagenicity assays in genotoxicity studies

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APPLICATION OF BACTERIAL MUTAGENICITY ASSAYS IN GENOTOXICITY STUDIES

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
IN DE WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. J.H.G.I. GIESBERS
VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN
IN HET OPENBAAR TE VERDEDIGEN
OP DONDERDAG 16 FEBRUARI 1984
DES NAMIDDAGS TE 2.00 UUR PRECIES

DOOR

ROBERT PIERRE BOS
GEBOREN TE AMERSFOORT

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CONTENTS

History of occupational cancer

The first description of occupational cancer is known from 1775 (see fig.1). It was Percival Pott who, being a proficient surgeon, had noticed that many chimney-sweepers had cancer of the scrotum and testicles. He supposed that this disease originated from a lodgement of soot in the rugae of the scrotum. In fact, by Pott's observation, the way was opened to the prevention of this disease and to the isolation and synthesis of the first known pure carcinogen. It took over a hundred years, however, before results of experimental research were published showing the causal relation between exposure to chemicals and cancer. In 1915, the Japanese researchers Yamagiwa and Ichikawa (1918) induced cancer on a rabbit's ear

C A N C E R

S C R O T I.

RAMAZINI has written a book de morbis artificum; the Colic of Poictou is a well-known diſtemper, and every body is acquainted with the diſorders to which painters, plummers, glaziers, and the workers in white lead, are liable; but there is a diſeaſe

eaſe as peculiar to a certain ſet of people which has not, at leaſt to my knowledge, been publickly noticed; I mean the chimney-ſweepers' cancer.

It is a diſeaſe which always makes its firſt attack on, and its firſt appearance in the inferior part of the ſcrotum; where it produces a ſuperficial, painful, ragged, ill-looking ſore, with hard and riſing edges. The trade call it the ſoot-wart. I never ſaw it under the age of puberty, which is, I ſuppoſe, one reaſon, why it is generally taken, both by patient and ſurgeon, for venereal, and being treated with mercurials, is thereby ſoon, and much exaſperated: in no great length of time, it pervades the ſkin, dartos, and membranes of the ſcrotum, and ſeizes the teſticle, which it enlarges, hardens, and renders truly and thoroughly diſtempered; from whence it makes its way up the ſpermatic proceſs into the abdomen, moſt frequently indurating, and ſpoiling the inguinal glands: when arrived within the abdomen, it affects

ſects ſome of the viſcera, and then very ſoon becomes painfully deſtructive

The fate of theſe people ſeems ſingularly hard, in their early infancy, they are moſt frequently treated with great brutality, and almoſt ſtarved with cold and hunger, they are thruſt up narrow, and ſometimes hot chimnies, where they are bruiſed, burned, and almoſt ſuffocated, and when they get to puberty, become peculiarly liable to a moſt noiſome, painful, and fatal diſeaſe.

Of this laſt circumſtance there is not the leaſt doubt, though perhaps it may not have been ſufficiently attended to, to make it generally known Other people have cancers of the ſame parts, and ſo have others, beſide lead-workers, the Poictou colic, and the conſequent paralyſis; but it is nevertheleſs a diſeaſe to which they are peculiarly liable; and ſo are chimney-ſweepers to the cancer of the ſcrotum and teſticles.

 If

If there be any chance of putting a ſtop to, or preventing this miſchief, it muſt be by the immediate removal of the part affected, I mean that part of the ſcrotum where the ſore is, for if it be ſuffered to remain until the virus has ſeized the teſticle, it is generally too late even for caſtration I have many times made the experiment; but though the ſores, after ſuch operation, have, in ſome inſtances, healed kindly, and the patients have gone from the hoſpital ſeemingly well, yet, in the ſpace of a few months, it has generally happened, that they have returned either with the ſame diſeaſe in the other teſticle, or in the glands of the groin, or with ſuch wan complexions, ſuch pale, leaden, countenances, ſuch a total loſs of ſtrength, and ſuch frequent and acute internal pains, as have ſufficiently proved a diſeaſed ſtate of ſome of the viſcera, and which have ſoon been followed by a painful death

If extirpation ever bids fair for the cure of a cancer, it ſeems to be in this caſe; but then the operation ſhould be immediate, and before the habit is tainted. The diſeaſe, in theſe people, ſeems to derive its origin from a lodgment of ſoot in the rugæ of the ſcrotum, and at firſt not to be a diſeaſe of the habit In other caſes of a cancerous nature, in which the habit is too frequently concerned, we have not often ſo fair a proſpect of ſucceſs by the removal of the diſtempered part; and are obliged to be content with means, which I wiſh I could ſay were truly palliative; but here the ſubjects are young, in general in good health, at leaſt at firſt, the diſeaſe brought on them by their occupation, and in all probability local; which laſt circumſtance may, I think, be fairly preſumed from its always ſeizing the ſame part all this makes it (at firſt) a very different caſe from a cancer which appears in an elderly man, whoſe fluids are become acrimonious from time, as well as other cauſes; or from

the ſame kind of complaint in women who have ceaſed to menſtruate. But be all this as it may, the ſcrotum is no vital organ, nor can the loſs of a part of it ever be attended with any, the ſmalleſt degree of inconvenience; and if a life can be preſerved by the removal of all that portion that is diſtempered, it will be a very good and eaſy compoſition; for when the diſeaſe has got head, it is rapid in its progreſs, painful in all its attacks, and moſt certainly deſtructive in its event.

Fig. 1 Description of the occurrence of cancer of the scrotum and testicles in chimney-sweepers by Percival Pott (1775).

by painting it with tar. The isolation of benzo(a)pyrene from coal tar was not described until 1933 (Cook, Hewett and Hieger).

At the end of the 19th century, skin cancers were observed not only in chimney-sweepers but also in workers exposed to shale oil, tar and products in several industries (Pott 1775, Syme 1835, Baum 1874, Volkmann 1874). The occurrence of lung tumours in the Schneeberg miners at Joachimstahl in Bohemia was reported in 1879 by Härting and Hesse. The tumours in all these cases had arisen at the place of contact in the skin or respiratory tract, but no occupational tumours were recognized in organs remote from the place of contact.

In 1895 at a congress of the German Surgical Society, Rehn a surgeon in Frankfurt-am-Main, reported three cases of bladder tumours in patients who had worked in the same factory, namely in the so-called fuchsin room, where at the most a total of 45 men were ever known to have worked. Thus, the incidence of this relatively uncommon disease was extraordinary high. These tumours were associated with exposure to aniline which gave rise to the introduction of the name "aniline cancer". Later on this turned out to be the wrong name when it was finally accepted that aniline was not the significant cause. Since the end of the 19th century, occupational bladder tumours were found more frequently in workers in aromatic amines (Leichtenstern 1898, Rehn 1904, 1906). Hueper et al. (1938) found tumours of the bladder in dogs treated with 2-aminonaphthalene (β-naphthylamine). Finally it became evident that 2-aminonaphthalene, 4-aminobiphenyl and benzidine were compounds producing bladder tumours in men (Clayson 1962, Koss et al. 1969).

In 1920 Oppenheimer supposed that it was a metabolite of benzidine or aniline which was especially active on the bladder epithelium. The possible role of metabolism in the carcinogenesis of aromatic amines has been discussed extensively by Bonser et al. (1951). It was found that in most of the experimental animals, tumours have been induced in various organs, yet the dog was the only animal in which bladder tumours appeared. Besides species-differences in the metabolism as a possible cause for the difference in response, it was found that the pH of the urine, which is acid in man and dog, can play a role. On the basis of their carcinogenicity experiments Walpole and his coworkers (1952) suggested an important working rule that; if a carcinogen does not produce tumours at the site of injection, then it is

not a direct or local carcinogen and that, if tumours are produced in organs remote from the site of administration, then the carcinogen must be a metabolite. Studies on the metabolism of carcinogenic compounds have played an important role in the elucidation of their mechanism of action. (Miller 1970, Weisburger and Williams 1975, Heidelberger 1975).

At present, 23 chemicals or groups of chemicals and 7 industrial processes or occupational exposures appear carcinogenic to humans on the basis of sufficient evidence from epidemiological studies (table 1). It can be noted that in 18 out of these 30 cases the main type of exposure is occupational. For hundreds of other chemicals some evidence of carcinogenicity was found in experimental animals (IARC Monographs).

TABLE 1

CHEMICALS, INDUSTRIAL PROCESSES AND OCCUPATIONAL EXPOSURES CAUSALLY ASSOCIATED WITH CANCER IN HUMANS

4-Aminobiphenyl
Analgesic mixtures containing phenacetin
Arsenic and arsenic compounds
Asbestos
Auramine manufacture
Azathioprine
Benzene
Benzidine
N,N-Bis(2-chloroethyl)-2-naphthylamine (Chlornaphazine)
Bis(chloromethyl)ether and technical-grade chloromethyl methyl ether
Boot and shoe manufacture and repair (certain occupations)
1,4-Butanediol dimethanesulphonate (Myleran)
Certain combined chemotherapy for lymphomas (including MOPP)
chlorambucil
Chromium and certain chromium compounds
Conjugated oestrogens
Cyclophosphamide
Diethylstilboestrol
Furniture manufacture
Isopropyl alcohol manufacture (strong-acid process)
Melphalan
Methoxsalen with ultra-violet A therapy (PUVA)
Mustard gas
2-Naphthylamine
Nickel refining
Rubber industry (certain occupations)
Soots, tars and oils
Treosulphan
Underground haematite mining (with exposure to radon)
Vinyl chloride

Mutagenesis and carcinogenesis

The first experimental laboratory data about a possible mechanism of carcinogenesis were published by Boveri (1914). According to this author the development of malignant tumours is connected with abnormal divisions of cell nucleus and chromosomes. This theory did not meet with the approval of well-known, contemporary pathologists. A new attempt to correlate mutagenesis and carcinogenesis was the formulation of the somatic cell mutation theory of cancer by Bauer (1928). According to his concept, mutation of a somatic cell should be the first step in the development of cancer. The theory of Bauer had a serious limitation: a mutational change in the genome of a cell is a very rapid process and could hardly account for the long period of latent carcinogenesis. The work of Berenblum (1941, 1949) suggested a multistage mechanism of carcinogenesis and instigated the concept of initiating and promoting agents. In principle, mutagenic agents have initiating properties. Recently, it was shown that a point mutation in an identified gene (oncogene) is responsible for the transformation of a normal human bladder cell in a tumour cell (Tabin et al. 1982, Reddy et al. 1982). Besides chemicals that are carcinogenic via interactions with DNA (genotoxic), there are substances that act via other (epigenetic) mechanisms.

Salmonella/mutagenicity assay

The development of bacterial systems for the detection of mutagenic properties of chemicals started in the early 1950's (Demerec et al. 1951, Hemmerly and Demerec 1955). At first it was supposed that testing of chemicals for mutagenicity with bacteria might be useful for the identification of potential anticancer agents, since many chemicals having mutagenic properties were both carcinogenic and carcinostatic in animal model systems. A few years later Iyer and Szybalski (1958) introduced the plate assay technique. This method appeared to be suited for the rapid evaluation of potential antineoplastic properties of large numbers of chemicals. A particular strain of *Escherichia coli* was used in these cases as the indicator organism. The majority of the mutagens identified in these studies were alkylating agents that were directly mutagenic. Because these bacterial systems tended to give false negatives for pre-mutagenic compounds which are converted into an active state in mammals, Gabridge and Legator (1969) developed a procedure in which

a microbial indicator was incorporated in an animal system to identify pre-mutagenic agents. This procedure in which the activation of pre-mutagens is performed by the mammalian metabolism, is commonly specified as "host-mediated assay". In 1973 Ames and coworkers introduced an *in vitro* test system for the screening of pre-mutagenic compounds. In the Ames assay the bioactivation process is mimicked by the addition of liver homogenates to the *Salmonella typhimurium* strains. Some of these strains were especially sensitive because of a deficient lipopolysaccharide barrier and because of lack of DNA excision repair. All of them were histidine auxotrophe mutants. The introduction of R plasmids in some of the strains increased their sensitivity to a broader range of chemicals (McCann et al. 1975, Levin et al. 1982a). Recently new tester strains which are especially sensitive in the detection of oxidative mutagens were introduced (Levin et al. 1982b, Levin et al. 1983). After ample evaluation of the method described by Ames et al. in 1975, revised methods for the Salmonella mutagenicity test were recently published by Maron and Ames (1983). *Salmonella typhimurium* strains used in this thesis are summarized in table 2.

TABLE 2

TESTER STRAINS

Additional Markers			Histidine Mutation		
LPS*	Repair	R-Factor	*hisC3076*	*hisD3052*	*hisG46*
+	+	-			*hisG46*
rfa	Δ*uvrB*	-	TA1537	TA1538	TA1535
rfa	Δ*uvrB*	+		TA98	TA100

*Lipopolysaccharide

All strains were originally derived from *Salmonella typhimurium* LT2. Wild-type genes are indicated by +. Abbreviations for the additional markers were: *rfa* = deep rough (defective lipopolysaccharide); Δ*uvrB* = deletion of the ultraviolet-repair B gene; R-Factor = plasmid pKM101 (increases error-prone DNA repair). (The strains were a gift from Dr. B.N. Ames, Berkeley, U.S.A.).

Besides the Salmonella/microsome assay many other so-called short-term assays have been developed for the detection of genotoxic effects, such as gene or point mutations, DNA repair, structural or numerical chromosomal aberrations and neoplastic transformation, in prokaryotic as well as eukaryotic cell systems (Hollstein et al. 1979).

Many studies have been performed on the validation of the Salmonella/microsome assay for its competence to predict carcinogenic properties of chemicals (Teranishi et al. 1975, McCann and Ames 1976, Purchase et al. 1976, Sugimura et al. 1976, Rinkus and Legator 1979, Rosenkranz and Poirier 1979, Simmon 1979, and Bartsch et al. 1980). 80 - 90% of known carcinogens tested in these studies appeared to be mutagenic. The percentage of the non-carcinogens being non-mutagenic turned out to be somewhat lower. All the validations showed, at first glance that, the Salmonella/microsome assay fails to detect a few classes of carcinogens e.g. dimethylamino- and hydrazine compounds, polychlorinated pesticides and some other non-alkylating chlorinated hydrocarbons (Ames and McCann 1981). However, some of the carcinogens that were reported to be negative, can now be detected using the new tester strains (Maron and Ames 1983). A question that still has to be solved however, is what proportion of chemicals selected at random, without prior knowledge as to their carcinogenic activity will give a positive result in this test. It should be noted that Burdette's analysis in 1955 of the lack of correlation between mutagenicity and carcinogenicity is explicable due to the methods available at that time for the mutagenicity screening. No notice was taken of the fact that most mutagens and carcinogens are inactive as such and need metabolic activation. Microorganisms applied in the mutagenicity test are insufficiently equiped with bioactivating enzymes. In this respect the incorporation of a 9000 g liver supernatant (S9 fraction) in the Salmonella/microsome assay appeared to be adequate. Although there now exists a reasonably good qualitative relationship between carcinogenicity and mutagenicity, it appears that a quantitative relationship is failing. Most likely this can be attributed to different metabolic activation processess *in vitro* and *in vivo* (Bartsch et al 1980).

The enormous number of chemicals that have to be tested for their carcinogenic potency is still increasing. Animal carcinogenicity assays are relatively expensive and laborious. So, there is a growing need for a cheap and reliable short-term bio-assay. Because none of the currently used short-term assays has an acceptable predictive value, it has been advised to use a

battery of these test systems to minimize the chance of scoring "false negatives". (ICPEMC 1982).

Bioactivation and bioinactivation

Xenobiotic compounds may enter the body by ingestion via food or drink, by inhalation or by absorption through the skin. They are distributed throughout the body depending on their physical chemical properties. Animal cells possess highly lipoid outer membranes and offer poor resistance to the penetration of lipophilic compounds. The excretory systems of cells are geared for the removal of water soluble-, rather than fat soluble compounds. Drug-metabolizing enzymes are able to convert lipophilic substances to more hydrophilic products by introduction of polar groups. High activities of these enzymes are found mainly in the liver, but also in the kidney, the lungs and the gastrointestinal tract (Weisburger and Williams 1975). Furthermore, the microbial flora present in the mammalian intestine may contribute to the metabolism of xenobiotics in the intact organism.

The metabolic transformations of xenobiotic substances are divided into two types of reaction, phase 1 and phase 2 reactions (Williams 1959). The phase 1 reactions primarily concern oxidative conversions, but also reductive and hydrolytic processes. The polar groups that arise by these reactions provide the necessary chemical structure for the phase 2 reactions. These are conjugations resulting, for instance, in the formation of glucuronides, sulfates, acetates or glutathione conjugates.

Primarily due to oxidative conversions - in a number of cases in combination with conjugative reactions - some chemicals can be activated to electrophilic species, which are able to react irreversibly with nucleophilic functions of cellular macromolecules, such as proteins, RNA and DNA.

In recent years, more and more evidence is furnished that conjugating processes can lead to more toxic reaction products. For instance, it is known that the N-sulfate, the N-acetate and the N-O-glucuronide of N-acetyl-2-aminofluorene are more reactive towards nucleic acids or proteins than N-hydroxy-N-acetyl-2-aminofluorene (Irving 1979). The carcinogenic effects of 7-methylbenz(a)anthracene and 7,12-dimethylbenz(a)anthracene are also related to their conversion into hydroxymethyl derivatives and subsequent formation of an ester bearing a good leaving group, which generates the ultimate electrophilic intermediate (Cavalieri et al. 1978, Brouns et al. 1980).

Conjugation with glutathione is usually considered as a reaction that de-activates electrophilic products. However, it has recently been discovered that conjugation of certain substances, such as 1,2-dihalogen compounds, with glutathione can lead to the formation of highly reactive metabolites (Rannug et al. 1978, van Bladeren et al. 1979, 1980).

It is also known that phase 2 conjugations can be followed by phase 1 oxidations, resulting in the production of very reactive metabolites. For instance, benzidine is first acetylated to diacetylbenzidine, after which oxidation yields the highly mutagenic N-hydroxy-N,N'diacetylbenzidine (Morton et al. 1979).

Metabolic activation in the Salmonella/mutagenicity assay

Ideally, in terms of carcinogenicity screening, drug-metabolizing enzyme preparations should be used in the Salmonella/microsome assay that are able to produce all active metabolites formed in man *in vivo*. For practical reasons it is impossible to use fresh human tissue in mass screening. Animal tissue able to produce the same metabolites *in vitro* as those formed in man *in vivo* would be an acceptable alternative. However, no such animal tissue is available. As a rule, rat liver S9 (9000 g supernatant) is incorporated in the test system. With respect to the prediction of the carcinogenicity in man, results from these experiments are subject to two extapolations: The extrapolation from *in vitro* to *in vivo* and from rat to man. Many examples of differences between *in vivo* and *in vitro* metabolism are known. In general, in the common Salmonella/microsome assay using the rat liver S9 fraction, phase 1 reactions prevail over the phase 2 reactions. In search after optimal experimental conditions approaching biotransformation *in vivo*, the balance between activating and inactivating enzymes seems to be very important. In this thesis we have tried to bridge the gap between biotransformation *in vitro* and *in vivo* by the application of isolated rat hepatocytes as the metabolizing system in the Salmonella/mutagenicity assay.

Diagnosis of carcinogenic hazards

Although therapy of some forms of human cancer has improved in recent years, there are still types of tumour for which treatment is relatively unsuccesful. Without neglecting this important area of anti-cancer research most attention should be paid to prevention. Because environmental factors,

in particular the chemical ones, are largely held responsible for the occurrence of human cancer (Fraumeni 1973, Higginson and Muir 1979, Fishbein 1982), minimization of the disease may be accomphished by reducing exposure to these factors. A survey of different strategies to fight the cancer problem is depicted in fig. 2. During the last two decades a great deal of progress has been made in the field of the identification of potentially cancer-causing chemicals. *In vitro* screening techniques have been developed which are very helpful to denounce the presence in the environment of man-made carcinogens, to avoid introduction of newly synthesized genotoxic chemicals and to produce safe alternatives if necessary. However, there still remains a background burden of natural and hardly eradicable factors, causing human cancer, related to personal habits and life-style. Among these are smoking tobacco and drinking of alcoholic beverages (Bridges et al. 1979, Obe and Ristow 1979, DeMarini 1983). Coffee and tea also contain substances mutagenic in *Salmonella typhimurium* (Nagao et al. 1979, Aesbacher et al. 1980). Genotoxic compounds have also been found among other natural substances, like flavonoids, anthraquinones and related compounds (Brown 1980). Mycotoxins like the powerful hepatocarcinogen aflatoxin B_1 produced by certain strains of *Aspergillus*, are common contaminants of several human foods (Campbell and Hayes 1976). Heating of protein-containing foodstuffs also results in the formation of very potent mutagenic substances (Commoner et al. 1978, Yoshida et al. 1980, Sugimura 1982). Intestinal bacteria are involved in the production of very potent nitrosamines from nitrite and amines e.g. present in food (Lintas et al. 1982). Although most of these examples may represent only a minor risk, which as such seems to be negligible, it has to be taken into account that a summation of many minor risks may stand for a potential hazard. This is particularly true when carcinogenic factors are also present in the working environment, in which levels of exposure can be relatively high.

To minimize genotoxic risk, when the working environment is contaminated with carcinogenic chemicals, the most obvious approach is to monitor the concentrations in the air. Such measurements, commonly referred to as "environmental monitoring", can give an indication of the external exposure. In addition, in many cases the possibility exists to measure the "internal exposure", through analysis of a biological specimen, e.g. blood, urine, expired air. This type of monitoring, so-called biological monitoring, gives

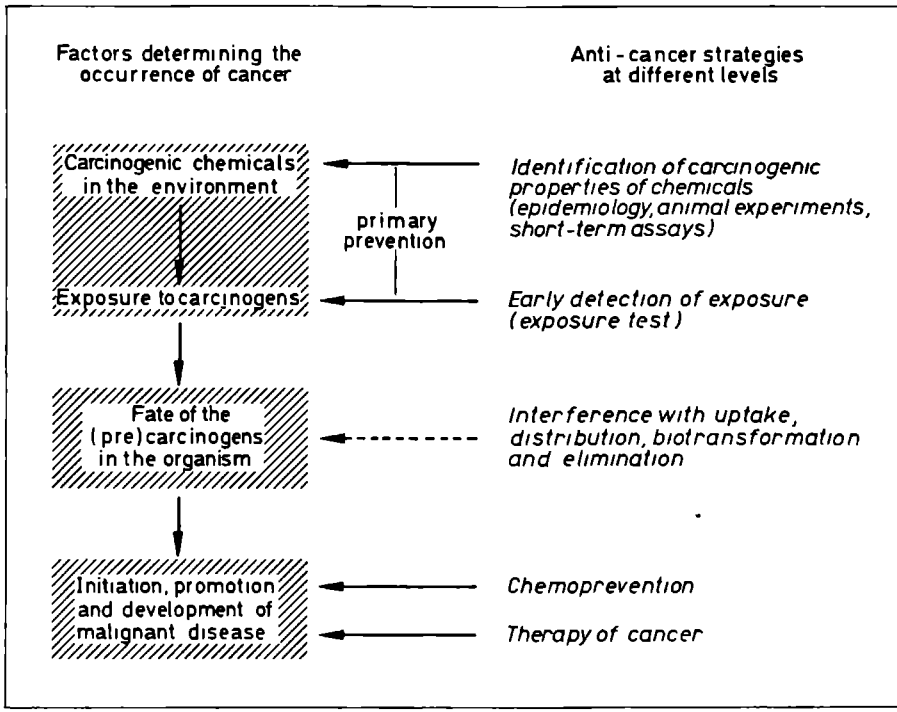

Fig. 2 A survey of different possibilities to fight the cancer problem.

a better approximation of the total load of the body and is more directly related to the health risk (Zielhuis, 1978). Whenever possible, selective methods are preferable in biological monitoring, in particular when the genotoxic chemical is known. Up to now only a limited number of tests has been evaluated. Add to this, that people may often be exposed to a mixture of chemicals (industrial chemicals, hobby-agents, food contaminants, drugs). In such cases as a first approach in the protection of health, non-selective tests may be of great value, because they have a signal function.

Recently, several reports have appeared on the detection of exposure to electrophilic compounds, including mutagens and carcinogens, by measurement of increased urinary thioether concentrations (Vainio et al. 1978, v. Doorn 1981). This non-selective assay is based on the ability of alkylating-, or otherwise covalently binding-compounds, to react with glutathione. The amount of thioethers excreted in urine may reflect the detoxication of such compounds. It has to be noted that in the case of either incomplete or not any detoxication via conjugation with SH-groups, false negative values are obtained.

The problem of false negative results seems to be inherent in the application of non-selective methods. To overcome this, it is important to combine several non-selective exposure assays which might be complementary to each other. (Analogous to the use of a combination of several short-term assays in the screening for carcinogenic potency of chemicals). Such a battery of assay procedures should include a selection of exposure tests, among which methods for detection of early health effects. Some examples of non-selective methods for detection of exposure to genotoxic compounds are described below.

- Measurement of products of the alkylation of proteins

  Most (potential) alkylating agents tend to react in the body with nucleophilic groups in e.g. peptides, proteins or nucleic acids. Alkylated proteins can be measured in blood for example by determining the degree of alkylation of hemoglobin amino acids in erythrocytes (Osterman-Golkar et al. 1976). Dependent upon the relationship between alkylation of proteins and alkylation of DNA such assays may be used for the detection of exposure to genotoxic substances. At this moment this relationship is not entirely clear.

- Measurement of DNA adducts

  Genotoxic alkylating agents or metabolites can react with various sites in DNA. Promising HPLC and/or immunochemical methods are being developed now for the detection of specific DNA adducts in e.g. blood cells (Baan et al. 1983).

- Analysis of chromosomal changes in human lymphocytes

  The observation of chromosomal aberrations in peripheral blood lymphocytes can be considered as an early effect of exposure to genotoxic agents, since most of the chemicals that cause visible chromosomal damage are known or suspected carcinogens (Vainio et al. 1981). This method is very laborious. For a review on this subject see Natarajan and Obe (1980).

- Analysis of sister chromatid exchanges (SCE)

  Sister chromatid exchanges are visualized intrachromosomal rearrangements of DNA helices. SCE's can be detected rather easily in human lymphocytes. Determination of the SCE-frequency seems to be a simpler, faster and less expensive technique of biological monitoring than determination of chromosomal aberrations. However, the molecular mechanism is still unclear and it is known that there are several drugs with a pronounced genotoxic activity which do not increase the SCE-frequency significantly over control values. (Lambert et al. 1982).

- Urinary mutagenicity assay

  Durston and Ames (1974) and Commoner et al. (1974) have suggested that a mutagenicity assay on urine samples would have great potential for screening the human population to detect environmental mutagenic and carcinogenic agents. It is one of the main objectives of the present thesis to evaluate the applicability of this assay.

OUTLINE OF THE THESIS

This thesis is composed of pre-published studies, most of which deal with the application of bacterial mutagenicity assays in the exploration of genotoxic hazards of chemical compounds. This extensive field of problems was approached at three different levels: by investigations *in vitro*, animal studies and studies on human subjects.

Part 1 includes studies of IN VITRO methods aimed at the detection of mutagenic properties of chemicals. In particular, attention was paid to the following aspects:

- Application of the Salmonella/microsome assay in combination with analytical chemistry-methods to identify mutagenic components in a complex chemical product (chapter 1).
- The question whether the excretion of glutathione conjugation products, which can be considered as an indication of detoxification of electrophilic intermediates, is also indicative of previous exposure to mutagens (chapter 2).
- Optimization of the *in vitro* mutagenicity assay by alterations in the metabolic activation system. Special attention was paid to the usefulness of intact isolated rat hepatocytes as the metabolic factor (chapter 3).

Part 2 describes the experimental studies IN VIVO with precarcinogenic compounds. Intact animals served as models to explore the possibilities and to refine the methods for the detection of genotoxic exposures by means of measurement of urinary mutagenicity. As substrates we used aromatic amines and azo dyes, in the biotransformation of which different pathways and various enzymes are involved (chapter 4).

Part 3 shows some typical applications of the Salmonella/mutagenicity assay for the detection of HUMAN EXPOSURE to environmental genotoxic compounds. This part of the thesis deals with the usefulness of the urinary mutagenicity assay as a tool in the evaluation of the genotoxic risk. Chapter 5 demonstrates the presence of mutagenicity in the urine of either active or passive smokers. Further, a study is presented on the urinary mutagenicity of nurses during occupational exposure to cytostatic drugs (chapter 6). Chapter 7 describes approaches to detect mutagenic substances in the work place by studying contaminations of the environment and by measuring urinary mutagenicity of workers involved in the preservative treatment of wood with creosote.

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PART 1
DETECTION OF MUTAGENIC PROPERTIES OF CHEMICAL PRODUCTS

Chapter 1

Application of the Salmonella/microsome assay

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Mutagenicity of creosote in the Salmonella/microsome assay

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Summary

Creosote (type P1) was assayed for mutagenicity in the Salmonella/microsome assay. It showed mutagenic properties towards *Salmonella typhimurium* TA1537, TA1538, TA98 and TA100 in the presence of S9 mix. This mutagenicity is probably caused by the presence of mutagenic aromatic hydrocarbons.

Creosote is well known as a pesticide in use for wood preservation. It is a mixture of oils that are separated in the distillation of coal tar and consists principally of liquid and solid hydrocarbons. It has a distinctive smell.

Production workers in the wood-preserving industry receive some level of exposure to creosote by inhalation of vapours or through skin contact. Apart from symptoms such as contact dermatitis and photosensitization, creosote oil may also cause skin cancer [5]. This is not unexpected because creosote may contain many PAHs that are present in coal tar, the source of creosote. It is striking, however, that creosote was recently denoted as non-mutagenic in bacterial assays [6]. For this reason we re-investigated the mutagenic properties of creosote (type P1) in the Salmonella/microsome assay.

Materials and methods

Chemicals

Benzo[*a*]pyrene and 1,2-epoxy-3,3,3-trichloropropane were purchased from Aldrich Europe (Beerse, Belgium). Creosote type P1 was from Cindu Chemicals BV

(Uithoorn, The Netherlands). D-Biotin and L-histidine-HCl were obtained from Sigma (St. Louis, U.S.A.). Nicotinamide adenine dinucleotide phosphate (NADP) disodium salt and glucose 6-phosphate (G-6-P) disodium salt were obtained from Boehringer (Mannheim, F.R.G.). Purified agar was purchased from Difco Laboratories (Detroit, U.S.A.) and nutrient broth No. 2 from Oxoid Ltd. (Basingstoke, Great Britain).

Mutagenicity testing

The mutagenicity test was performed according to Ames et al. [1] with *Salmonella typhimurium* TA1535, TA1537, TA1538, TA98 and TA100. We used Oxoid nutrient broth instead of Difco nutrient broth.

Rat-liver S9 (9000 *g* supernatant) fractions were prepared from male Wistar rats, pretreated with Aroclor 1254. S9 mix contained 0.1 ml S9 per ml.

A portion of rat-liver S9 was centrifuged at 105000 *g* for 1 h. The resulting pellet was resuspended in 0.15 M KCl. The microsome mix contained 0.1 ml of this suspension per ml.

In one experiment the epoxide hydratase inhibitor 1,2-epoxy-3,3,3-trichloropropane (TCPO) was added at 2.3 μmoles/plate [2].

Results

Mutagenicity of creosote

The mutagenicity of coal-tar creosote P1 towards *Salmonella typhimurium* strains TA1537, TA1538, TA98 and TA100 is shown in Fig. 1. Strain TA1535 appeared to be insensitive to the mutagenic activity of creosote. With the other strains the mutagenic activity became evident after the addition of S9 mix. We studied the toxicity of creosote for *Salmonella typhimurium* with strains TA98 and TA100. At 50 μg/plate, the survival of TA98 and TA100 was 100%.

The presence of mutagenic polycyclic aromatic hydrocarbons in creosote

Oesch et al. [4] reported that inhibition of epoxide hydratase in rat-liver microsomes leads to a dramatic potentiating of the mutagenic effect of benzo[*a*]pyrene in *Salmonella typhimurium* TA1537. This effect was explained by accumulation of the intermediate benzo[*a*]pyrene-4,5-oxide, which is mutagenic towards *Salmonella typhimurium* TA1537 [7].

In Fig. 2b we show that addition of the epoxide hydratase inhibitor 1,1,1-trichloropropane-2,3-oxide to liver microsomes from Wistar rats pretreated with Aroclor 1254 substantially enhanced the mutagenic effect of benzo[*a*]pyrene. This effect was also found when creosote was plated instead of benzo[*a*]pyrene (Fig. 2a). From these results we may conclude that polycyclic aromatic hydrocarbons are, at least partly, responsible for the mutagenicity of creosote P1.

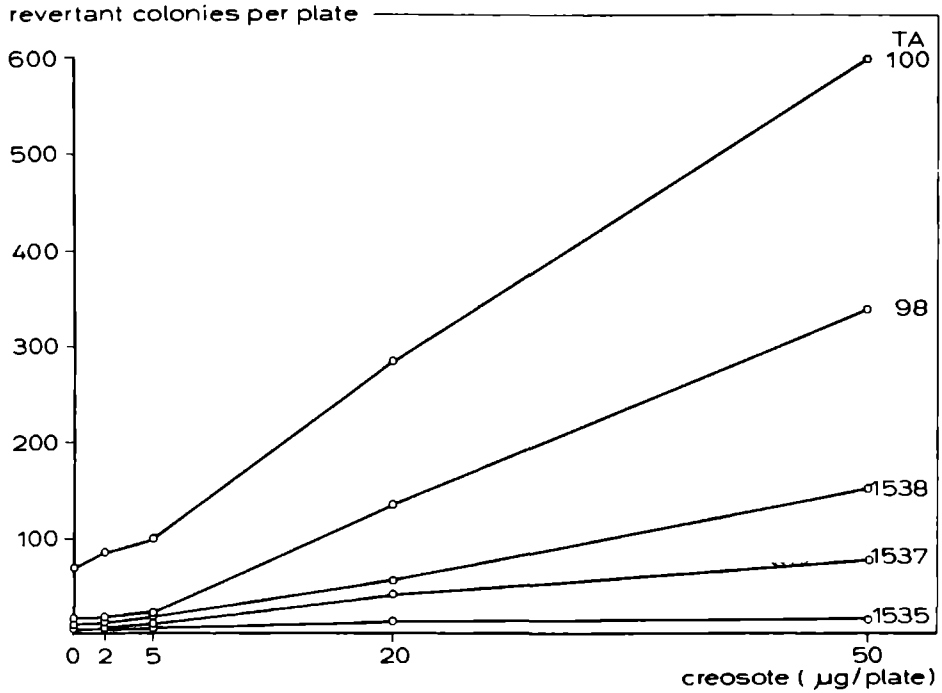

Fig. 1. Number of revertant colonies per plate of 5 *Salmonella typhimurium* strains as a function of the dose of creosote. Each point represents the average of the counts of 3 plates. Liver 9000 *g* supernatant (S9 mix) was added.

Discussion

Our results* (Fig. 1) are in contrast with results of examinations of the mutagenic activity of coal-tar creosote P1 conducted by Litton Bionetics Ltd. and presented in a Report of the U.S. Department of Agriculture [6]. This report states that both non-activation and activation test results were all negative. The present demonstration of a mutagenic effect of creosote in *Salmonella typhimurium* indicates a genetic hazard for humans exposed to this substance and correlates well with the carcinogenicity data mentioned by Sax [5].

Mutagenicity of epoxides towards *Salmonella typhimurium* TA1537 is only known for some epoxides of polycyclic aromatic hydrocarbons (benzo[*a*]pyrene-4,5-oxide, dibenz[*a,h*]anthracene-5,6-oxide and chrysene-5,6-oxide) [3, 7]. In addi-

* After completion of this paper we were notified by Dr. D.J. Brusick (personal communication) that positive data have now likewise been obtained at Litton Bionetics.

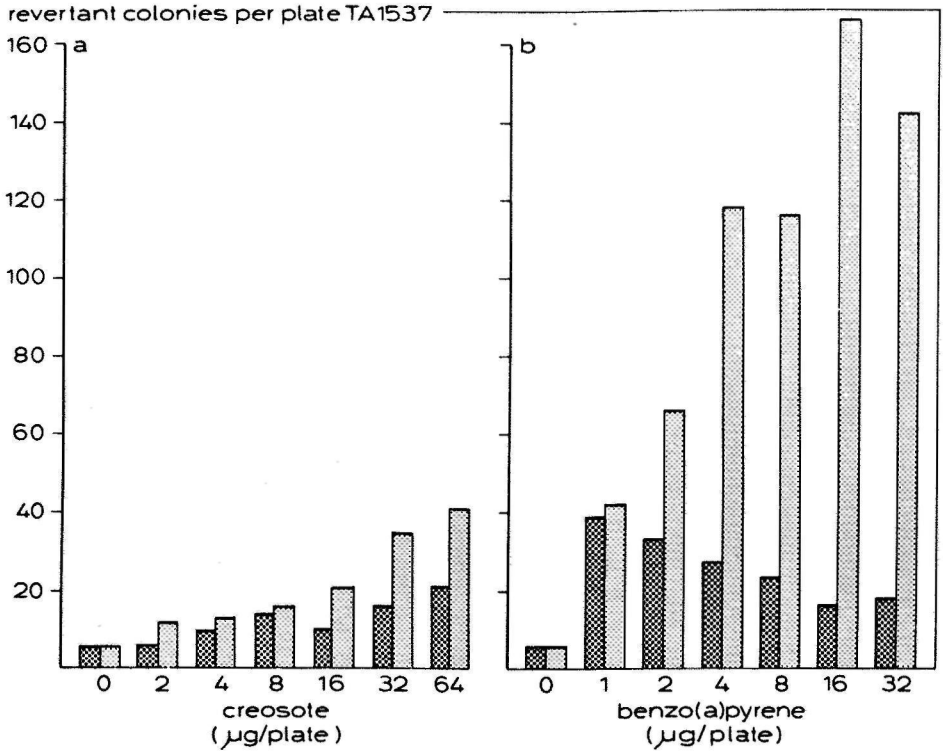

Fig. 2. The influence of the epoxide hydratase inhibitor TCPO on the number of *Salmonella typhimurium* TA1537 revertant colonies per plate as a function of the dose of creosote (a) or benzo[*a*]pyrene (b). The dark hatched bars represent the number of revertant colonies in the absence of TCPO. Creosote and benzo[*a*]pyrene were plated in the presence of microsome mix. The values are averages of determinations in triplicate.

tion, a number of polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene, dibenz[*a,c*]anthracene, dibenz[*a,h*]anthracene, 3-methylcholanthrene and 7,12-dimethylbenzanthracene are mutagenic towards *Salmonella typhimurium* TA1537 in the presence of S9 mix [3]. From this and from the results presented in Fig. 2 it is tempting to conclude that mutagenic polycyclic aromatic hydrocarbons are present in creosote P1. Further studies are in progress to identify the chemical structure of the mutagen(s) present in creosote P1.

Acknowledgement

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THE PRESENCE OF THE MUTAGENIC POLYCYCLIC AROMATIC HYDROCARBONS BENZO(A)PYRENE AND BENZ(A)ANTHRACENE IN CREOSOTE P1

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SUMMARY

Several fractions of creosote P1 separated by TLC showed mutagenicity towards *Salmonella typhimurium* TA98. This mutagenicity is probably caused by the presence of mutagenic aromatic hydrocarbons. The mutagenic polycyclic aromatic hydrocarbons, benzo(a)pyrene and benz(a)anthracene were detected in concentrations of 0.18 and 1.1 per cent, respectively. Because these compounds are probably not essential for the wood-preserving properties of creosote, a more selective composition of the product should be considered.

INTRODUCTION

In a previous paper we have shown that creosote (type P1), well known as a pesticide in use for wood preservation, has mutagenic properties in the Salmonella/microsome assay (4). In that study, the influence of the epoxide hydratase inhibitor TCPO on the mutagenicity of creosote towards *Salmonella typhimurium* TA1537 suggests the presence of mutagenic polycyclic aromatic hydrocarbons. Our present data actually show the presence of the mutagenic PAH's benzo(a)pyrene and benz(a)anthracene in creosote by means of a combination of mutagenicity testing and analytical methods.

Accepted for publication by Mutation Research

Chemicals

1,2-epoxy-3,3,3-trichloropropane was purchased from Aldrich Europe (Beerse, Belgium). Benz(a)anthracene was obtained from ICN Pharmaceuticals, Inc. Creosote type P1 was from Cindu Chemicals B.V. (Uithoorn, The Netherlands). D-Biotin, L-histidine-HCl, benzo(a)pyrene, nicotinamide adenine dinucleotide phosphate (NADP) disodium salt and glucose-6-phosphate (G-6-P) disodium salt were obtained from Sigma (St. Louis, U.S.A.). Purified agar was purchased from Difco Laboratories (Detroit, U.S.A.) and nutrient broth No. 2 from Oxoid Ltd. (Basingstoke, Great Britain).

Thin-layer chromatography

200 µl of a solution of creosote in ethanol (10 mg/ml) was applied to a TLC-plate in a continuous streak with a line applicator.

Glass plates (20 x 20 cm) coated with a 0.25 mm thick layer of silicagel 60 obtained from Merck (Darmstadt, F.R.G.) were employed. Pentane/diethylether (95 : 5; v/v) was used as solvent system. Benzo(a)pyrene and benz(a)anthracene were visualized on the TLC-plate under UV-light of 254 nm. Some fractions on the TLC-plate were scraped off. The contaminated silicagel was suspended in 5 ml of acetone. Silicagel residues were removed by centrifugation. The acetone was evaporated under nitrogen at 60°C and the residues were dissolved in 0.25 ml of methanol or 1 ml of dimethylsulphoxide.

Mutagenicity testing of the TLC fractions

The mutagenicity test was performed according to Ames et al. (2). Samples of 0.1 ml of the solution of the residues in DMSO were added per plate and assayed for mutagenicity with the *Salmonella typhimurium* strain TA98 in the presence of S9 mix. S9 was prepared from male Wistar rats, pretreated with Aroclor 1254. These fractions were also tested for mutagenicity in *Salmonella typhimurium* TA1537 after activation by mix made from liver microsomes of male Wistar rats pretreated with Aroclor 1254. In this experiment the influence of the addition of the epoxide hydratase inhibitor 1,2-epoxy-3,3,3-trichloropropane (TCPO) (2.3 µmoles/plate) was studied (3,4). Rat liver microsomes were prepared by centrifugation of rat-liver S9 at 105000 *g* for 1 h.

High-performance liquid chromatography (HPLC)

Aliquots of the solubilized residue were filtrated (0.22 µm) and injected into the 0.020 ml sample loop of a Pye Unicam HPLC equipped with a 3 XP Pump, a variable wavelength detector and a 10 mV Kipp recorder. The stainless steel column was 150 mm long (i.d. 4.6 mm) and filled with Lichrosorb RP 18, particle size 5 µm. The flow rate was 1.00 ml/min and the wavelength was adjusted to 254 nm. Simultaneously with sample injection, the following solvent programme was commenced: 10 min linear gradient from 100% solvent A (70% aquapur, 30% methanol; 80 µl acetone/l) to 100% solvent B (10% aquapur, 90% methanol; 80 µl acetone/l), followed by 20 min solvent B.

Fluorometric assay (qualitative)

The methanol samples supposed to contain benzo(a)pyrene, or benz(a)anthracene were diluted and measured with a Perkin-Elmer 650-40 spectrofluorometer at an excitation wavelength of 380 or 285 nm and an emission wavelength of 433 or 391 nm for benzo(a)pyrene and benz(a)anthracene, repectively. The excitation and emission slits were 5 and 10 nm, respectively. Quinine.HBr in 0.1 N H_2SO_4 was used for the calibration of the fluorometer.

Quantification of benzo(a)pyrene and benz(a)anthracene

A Hewlett-Packard HP1084B high-performance liquid chromatograph equipped with a variable-volume injector and a fluorescence spectrometer Model 3000 (Perkin-Elmer) was used. The stainless steel column was 150 mm long i.d. 4.6 mm and filled with Lichrosorb RP18, particle size 5 µm. The oven temperature was 37°C and the injection volume was 10 µl. The excitation wavelength was 380 and 285 nm, the emission wavelength was 433 and 391 nm for benzo(a)pyrene and benz(a)anthracene, respectively. The excitation slit was 10 nm, the emission slit 5 nm. The solvent programme was as described under HPLC. Dilutions of total creosote, benzo(a)pyrene and benz(a)anthracene in methanol were injected.

RESULTS

Different mutagenic fractions of creosote

After the application of creosote in a continuous streak to a TLC-plate, several fractions were recognized under UV-light of 254 nm. Fractions were

scraped off and tested for mutagenicity towards *Salmonella typhimurium* TA98 in the presence of S9 mix. The results are shown in figure 1. In a

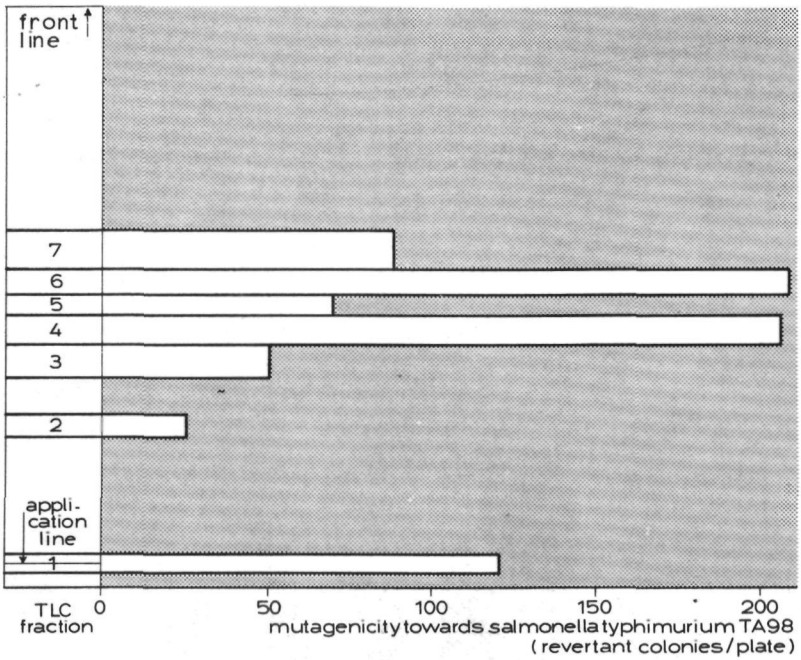

Fig. 1 Number of revertant colonies per plate of Salmonella typhimurium TA98 as a function of the TLC fraction. The mutagenicity was detected in the presence of S9 mix. Mutagenicity values represent averages of counts of 3 plates.

preliminary experiment no obvious mutagenicity was detected in the area between fraction 1 and fraction 3 and the area between fraction 7 and the finish line. It was observed that the fractions 1,4 and 6 show most of the mutagenicity of creosote. In figure 2 it is demonstrated that addition of the epoxide hydratase inhibitor 1,1,1-trichloropropane-2,3-oxide to liver

microsomes enhanced the mutagenic effect of the TLC-fractions. As reported earlier (4) such results may indicate the presence of mutagenic polycyclic aromatic hydrocarbons. We may conclude that the highly mutagenic fractions 1,4 and 6 contain different polycyclic aromatic hydrocarbons.

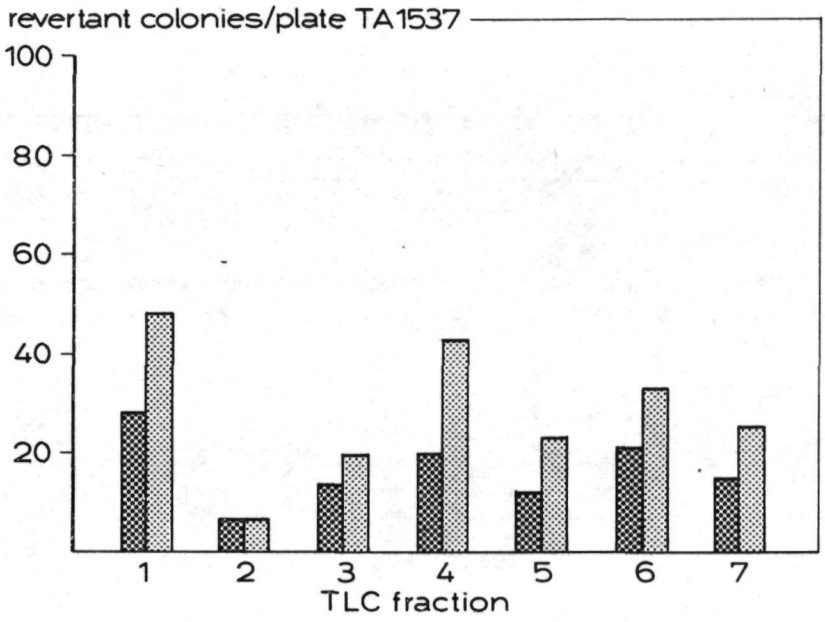

Fig. 2 Influence of the epoxide hydratase inhibitor TCPO on the number of Salmonella typhimurium TA1537 revertant colonies per plate after testing of the different TLC fractions (see fig. 1). The dark hatched bars represent the number of revertant colonies in the absence of TCPO. The values are averages of determinations in triplicate.

Identification of benzo(a)pyrene and benz(a)anthracene

Benzo(a)pyrene and benz(a)anthracene were found on TLC-plates under

UV-light of 254 nm having Rf values of 0.43 and 0.50 respectively. On the basis of these Rf values it might be possible that the TLC-fractions 4 and 6 contain benzo(a)pyrene and benz(a)anthracene, respectively. To support this assumption the TLC-fractions 4 and 6 were tested for the presence of these compounds.

The chromatographic behaviour of the TLC-fractions studied with the HPLC method as described under materials and methods, suggested the presence of benzo(a)pyrene in TLC-fraction 4 and benz(a)anthracene in TLC-fraction 6.

In addition the fluorescence characteristics of the methanol solutions derived from the TLC-fractions 4 and 6 were compared with those of benzo(a)-pyrene and benz(a)anthracene. By scanning at an excitation wavelength of 380 nm or an emission wavelength of 433 nm it was found that the TLC-fraction 4 showed the characteristics of benzo(a)pyrene (fig. 3a,b).

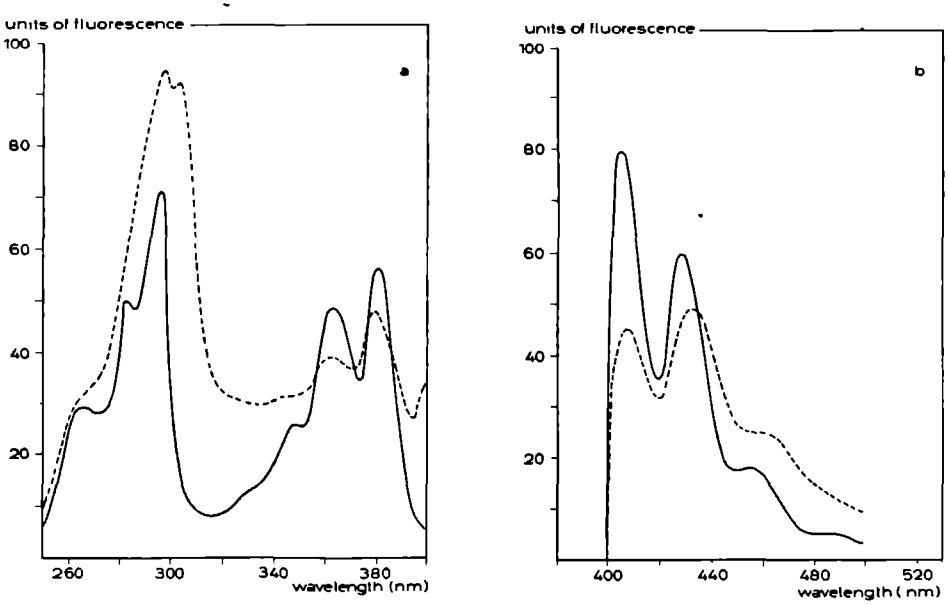

Fig. 3 Excitation (a) and fluorescence (b) spectra of TLC-fractions 4 (see fig. 1) from pure benzo(a)pyrene (solid lines) and from creosote (dotted lines).

In a similar way the presence of benz(a)anthracene was confirmed in TLC-fraction 6 at an excitation wavelength of 285 nm or an emission wavelength of 391 nm (fig. 4a,b).

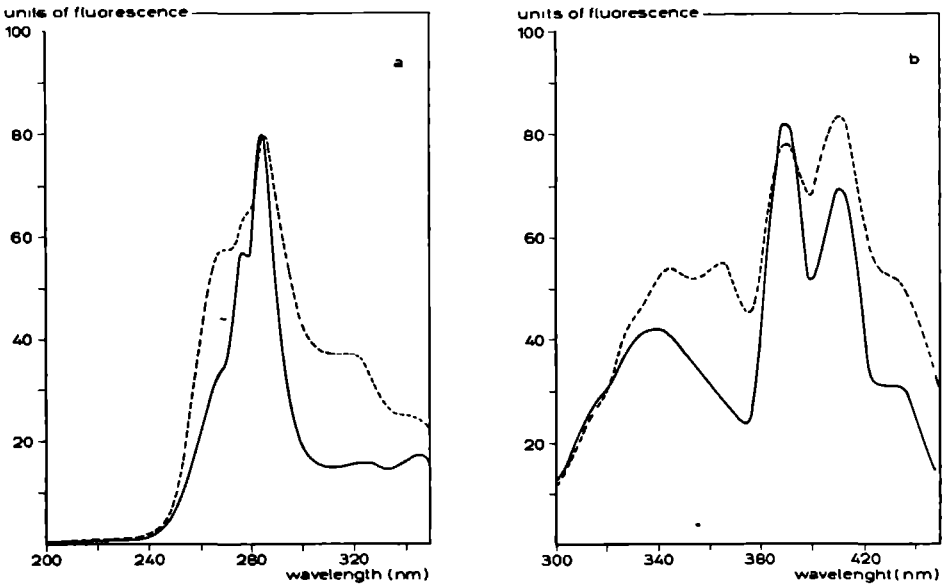

Fig. 4 Excitation (a) and fluorescence (b) spectra of TLC-fractions 6 (see fig. 1) from pure benz(a)anthracene (solid lines) and from creosote (dotted lines).

Quantification of benzo(a)pyrene and benz(a)anthracene

Concentrations of benzo(a)pyrene and benz(a)anthracene in creosote type P1 were assayed with HPLC equipped with fluorescence detection. In case of benzo(a)pyrene an excitation wavelength of 380 nm was used; the emission was measured at 433 nm. It was calculated that creosote contains 1.8 gram of benzo(a)pyrene per kg. To determine the concentration of benz(a)anthracene

an excitation wavelength of 285 nm was used. Emission was measured at 391 nm. It was calculated that creosoot type P1 contains 11 gram of benz(a)anthracene per kg.

DISCUSSION

Our previous suggestion that polycyclic aromatic hydrocarbons might, at least partly, be responsible for the mutagenicity of creosote (4) has been confirmed by the present results, which clearly show that creosote type P1 contains substantial concentrations of benzo(a)pyrene and benz(a)anthracene, 0.18 and 1.1 per cent, respectively. We were not able to identify the mutagen(s) present in the TLC-fraction around the application line (fig. 1). This mutagenicity must be due to the presence of more polar aromatic hydrocarbons.

Creosote P1 consists of several distillation fractions from coal tar. Lijinski et al. (5) demonstrated the presence of many polycyclic aromatic hydrocarbons in coal tar. Analysis with gas chromatography showed concentrations of benzo(a)pyrene and benz(a)anthracene of about 1.9 and 6.6 g/kg, respectively. Creosote type P1 is much richer in materials boiling below 355^{o}C and much leaner in residual materials (19%) than coal tar (65%) (1). Therefore, considering the boiling points of 495 and 438^{o}C of benzo(a)pyrene and benz(a)anthracene, respectively, much lower concentrations in creosote type P1 should be expected. However, the concentrations we detected were equal or even higher. Because we think that benzo(a)pyrene and benz(a)anthracene are not essential for the wood-preserving action of creosote, a more selective composition of the product, should be considered in particular with regard to the residual fractions.

ACKNOWLEDGEMENTS

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Chapter 2

Is excretion of glutathione conjugation products indicative of mutagenic exposure?

Arch. Toxicol. 43, 293–304 (1980)

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TOXICOLOGY

Effect of Toluene and Xylenes on Liver Glutathione and Their Urinary Excretion as Mercapturic Acids in the Rat

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Abstract. Administration of toluene and xylenes to rats caused a decrease in liver glutathione concentration. The effect was most pronounced after the administration of o-xylene. 26% of the initial glutathione level was found three hours after treatment with o-xylene (4.0 mmoles/kg).

No in vitro conjugation of o-xylene with glutathione was observed, neither spontaneously nor in the presence of 105,000 *g* supernatant from rat liver homogenate, containing glutathione S-transferases. Thus, a metabolite of o-xylene, which is not formed during incubation with 105,000 *g* supernatant, reacts with glutathione.

A thioether was isolated from urine of rats given o-xylene; the compound was identified as o-methylbenzyl mercapturic acid by GC-MS and NMR. Chromatographic evidence was found for the presence of benzyl mercapturic acid in the urine of toluene-treated rats. The amounts of mercapturic acids excreted in the urine after administration of toluene, p-xylene, m-xylene, and o-xylene were 0.4–0.7, 0.6, 1.3, and 10–21% of the dose, respectively.

These results demonstrate the involvement of a thusfar unknown pathway in the biotransformation of toluene and xylenes.

Key words: Toluene and Xylenes metabolism – Glutathione – Mercapturic acids.

Introduction

Benzene, toluene and xylenes are major constituents of coal tar naphtha and crude oil. There is a widespread use of these aromatic hydrocarbons as solvents. Occupational exposure to benzene, toluene and xylenes is very common. An association between long-term exposure to benzene and leukemia in man has been described (Mallory et al., 1939; Vigliani and Saita, 1964; Sanita, 1973). For this reason there have been many attempts to lower exposure levels.

Today there is a tendency to substitute benzene by toluene and xylenes, which are regarded less toxic and are used as "safe" replacement in several industries. Thusfar,

however, there is a paucity of knowledge on the chronic toxicity of toluene and particularly of the xylenes (Dean, 1978).

The presumed safety of toluene and xylenes is mainly based on metabolic considerations. Unlike unsubstituted aromatic compounds toluene and xylenes are thought to be metabolized almost exclusively by side-chain oxidation (Williams, 1959; Ogata, 1970) and not via aromatic epoxidation. Thus, reactive electrophiles that are responsible for many toxic effects, are believed to be formed only to a minor extent from toluene and xylenes. Bakke and Scheline (1970) demonstrated that in rat 0.4 to 1.1% of a dose of toluene is hydroxylated to o- and p-cresol. Further, they found dimethylphenols in the urine of rats after administration of p-, m-, and o-xylene (1.0, 0.9, and 0.1% of the doses, respectively). Small amounts of o-cresol were also found in the urine of printing workers exposed to toluene (Angerer, 1979). These findings suggest that epoxidation of the aromatic nucleus is a minor pathway in the metabolism of toluene and xylenes.

Aromatic epoxides, however, not only rearrange spontaneously to phenols, but can also be metabolized to dihydrodiols by a reaction with water or to glutathione conjugates by combining with glutathione (Jollow et al., 1974). Glutathione conjugates commonly appear in urine as mercapturic acids or cysteine conjugates (Chasseaud, 1976). Such thioethers also appear in urine as a result of detoxication of reactive, electrophilic substances other than epoxides (Johnson, 1965; Jones, 1973). Elevated urinary levels of thioether compounds may, therefore, generally be connected with the elimination of potentially alkylating agents.

In the current investigation we studied the urinary excretion of thioethers and the concomitant effects on hepatic glutathione concentrations in the rat after administration of toluene and xylenes, in order to ascertain whether or not these benzene derivatives are eliminated exclusively via "safe" metabolic routes.

Materials and Methods

Chemicals. Triethylamine, sodium borohydride, toluene, o-, m-, and p-xylene were obtained from Merck-Schuchard (Hohenbrunn, FRG). Glutathione and 5,5′ dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Boehringer (Mannheim, FRG). α-Bromo-o-xylene and S-benzyl-L-cysteine were obtained from Aldrich-Europe (Beerse, Belgium) Amberlite, type XAD-2 was obtained from Serva (Heidelberg, FRG).

Animals. Adult male Wistar rats, weighing 180–220 g, were purchased from TNO (Rijswijk, The Netherlands). The animals were housed in individual stainless steel metabolism-cages, with free access to water and food (Hope Farms, Woerden, The Netherlands). Urine was collected daily and frozen until required.

Toluene and xylenes were administered intraperitoneally in 0.5 ml arachis oil. In one experiment rats were given toluene orally in 1.0 ml propylene glycol.

Thin Layer Chromatography (TLC). Urine was adjusted to pH 2 with 5 N HCl. The samples were applied to Amberlite filled columns (bed volume 20 ml) The absorbed material was eluted with acetone The eluate was evaporated to dryness and the residue was dissolved in aqua dest. The aquous solution was extracted with ethyl acetate. The ethyl acetate layers were evaporated to dryness, the residue dissolved in methanol and applied to TLC-plates. Glass plates (5 x 20 cm) coated with a 0.25 mm thick layer of silica gel 60 F254 obtained from Merck (Darmstadt, FRG) were used.

Three solvent systems were used in the TLC experiments
a) butanol water ammonia (25%) (3 1 1, v/v),
b) methylene chloride methanol acetic acid (10 4 1, v/v),
c) butanol water acetic acid (3 1 1, v/v)
The spray reagent $K_2Cr_2O_7$ (0 1 M in glacial acetic acid, 1 1, v/v) followed by $AgNO_3$ (0 1 M) was applied to developed plates for the detection of divalent sulfur

Spectra Nuclear magnetic resonance spectra were obtained with compounds dissolved in CD_3OD or $CDCl_3$ using a 90 MHz Bruker LH 90 NMR spectrometer and tetramethylsilane as an internal reference (δ = 0 00 ppm)

Mass spectra were obtained with a V G Micromass 70–70F mass spectrometer at 160° C ion source temperature and 70 eV The compounds were introduced by direct probe insertion at 40° C, ion source pressure 1 3 x 10^{-4} Pa

Determination of Liver Glutathione Rats were killed at 2 00 p m by decapitation under light ether anaesthesia three hours after having received the various compounds The livers were perfused in situ with saline, rapidly removed and kept in ice Liver portions (0 4–0 6 g) were homogenized in 20 volumes of ice cold 0 15 M KCl For the estimation of reduced glutathione, 2 ml of homogenate were deproteinized by the addition of 3 ml of a reagent consisting of 120 g NaCl, 6 68 g metaphosphoric acid and 0 8 g EDTA dissolved in 400 ml aqua dest After centrifugation at 40,000 *g* for 20 min, 0 5 ml of the supernatant was added to 2 0 ml of 0 3 M Na_2HPO_4 2 H_2O solution SH groups were assayed by the method described by Ellman (1959), 0 2 ml of a DTNB solution (0 4 mg/ml 1% sodium citrate) was added and the absorbance at 412 nm was read immediately after mixing

Assay of Glutathione Conjugation in Vitro Both enzymatic and spontaneous conjugation of o xylene with GSH were measured in vitro The amount of conjugated product was assayed indirectly by determining the GSH consumption The incubation mixture consisted of 2 0 ml $KH_2PO_4 - Na_2HPO_4$ buffer (0 1 M, pH = 8 0), 0 25 ml GSH (12 mM), and 0 50 ml of 105,000 *g* rat liver supernatant The reaction was started by the addition of o-xylene in 0 10 ml ethanol, final concentrations of o-xylene were 0, 0 5, 1 0, and 2 0 mM After 10 min incubation at 37° C the reaction was stopped by the addition of 4 0 ml of the deproteinizing reagent, mentioned above After centrifugation at 40,000 *g* for 20 min the GSH concentration in the supernatant was determined

Determination of Urinary Thioether Excretion The urinary excretion of thioethers was determined by a modification of the procedure described previously (van Doorn et al, 1979)

Urine samples (0–24 h) were diluted about twofold and centrifuged at 3,000 *g* for 5 min Aliquot portions of 5 ml were transferred into glass stoppered tubes, the pH was adjusted to 1 5–2 0 with 4 N HCl and 8 0 ml ethyl acetate was added The layers were shaken vigourously for 15 min using a shaking apparatus The layers were separated by centrifugation at 3,000 *g* for 5 min The extraction procedure was repeated with another 8 0 ml of ethyl acetate The ethyl acetate layers were collected and subsequently evaporated to dryness using a rotary evaporator The residue was taken up in 2 0 ml aqua dest

The SH concentration was determined according to Ellman (1959) a 0 25 ml aliquot of the aqueous solution was added to a mixture consisting of 2 0 ml 0 5 M Sorensen phosphate buffer (pH 7 1) and 0 3 ml DTNB solution (0 4 mg DTNB per ml 1% sodium citrate solution)

Absorbances were read at 412 nm Corrections were made for the attribution to the absorbance by the colour of the urine extract and the DTNB solution

Alkaline hydrolysis was performed on 1 0 ml of the rest of the urine extract in brown glass stoppered test tubes by the addition of 0 5 ml of 4 N NaOH, saturation of the resulting solution with N_2, and keeping the closed tubes in a boiling water bath for 50 min After hydrolysis the tubes were cooled in ice for 10 min Then 0 5 ml 4 N HCl was added under mixing Exactly 5 min later the SH-concentration was determined as described above The difference in SH concentration after and before alkaline hydrolysis reflects the thioether concentration

Further corrections for the presence of disulfides in the extracts were found to be unnecessary

Determination of Creatinine The creatinine concentration of each urine sample was determined as described by Gorter and De Graaff (1955) The urinary levels of thioether compounds were expressed as mmoles –SH/mole creatinine

Synthesis of Mercapturic Acids. N Acetyl S benzyl-L-cysteine (benzylmercapturic acid) was prepared by the reaction of S benzyl-L-cysteine with acetic anhydride. Ten mmoles (2 43 g) S-benzyl L cysteine was solubilized in 10 ml 1 N NaOH and cooled in ice Fifteen mmoles (1 5 ml) acetic anhydride was slowly added to the solution in ten fractions of 0.15 ml under continuous stirring After every addition 20 ml of 1 N NaOH was added. The temperature of the mixture was allowed to rise to room temperature The pH was adjusted to 1.5 and the water layer was extracted with ethyl acetate The extract was dried (Na_2SO_4) and evaporated to afford a white crystallizing oil which was re crystallized from hexane/ethanol. The m.p of the compound was 144–145° C (Clapp et al., 1970, reported 147° C).

N-Acetyl S-(o-methylbenzyl)-L-cysteine was prepared by a reaction of α-bromo-o-xylene with N-acetyl-L cysteine. Five mmoles (817 mg) N acetyl-L-cysteine, 5 mmoles (0 685 ml) α-bromo o-xylene and 45 mmoles (6.18 ml) triethylamine in the presence of a catalytic amount of NaI were refluxed in 30 ml dioxane for 3 h. The compound that crystallized from the solution was filtered off The filtrate was collected in 170 ml ice cold water. The mixture was extracted several times with ether. Subsequently, the water layer was adjusted to pH 1 and extracted twice with one volume of ether. The ether layers were combined, dried (Na_2SO_4) and evaporated to afford a residue which was crystallized and re-crystallized several times from hexane/ethanol. The m.p. of the crystalline white compound, which was identified as N-acetyl S-(o methylbenzyl)-L-cysteine by NMR and GC-MS (Table 3, Fig. 2), was 153° C.

Results

Depletion of Hepatic Glutathione

The administration of toluene and the xylenes to rats resulted in a decrease in hepatic glutathione within 3 h after treatment. It is shown in Table 1 that, when these solvents are given in equal doses (4 mmoles/kg), o-xylene was most effective causing a decrease of about 75%, followed by m-xylene, p-xylene, and toluene. The effect of o-xylene on the hepatic glutathione concentration as a function of the dose is presented in Fig. 1. Almost maximal depletion was reached after 4 mmoles/kg.

24 h after the administration of the highest dose of o-xylene the GSH level was found to be in the normal range again.

Theoretically, conjugation of o-xylene with glutathione can take place by one or more of the following mechanisms: (1) spontaneously, by non-enzymatic conjugation;

Table 1. Hepatic glutathione levels in the rat after the administration of toluene and xylenes

Treatment[a]	GSH concentration[b] (μmoles/g liver)
Control	7.6 ± 0.3
Toluene	5.5 ± 0.3
p-Xylene	4.6 ± 0.1
m-Xylene	4.3 ± 0.2
o-Xylene	2.0 ± 0.2

[a] The compounds were administrated intraperitoneally in arachis oil (4.0 mmoles/kg body weight)
[b] Liver glutathione concentrations were determined 3 h after the administration of the compounds (mean values ± SEM of four determinations)

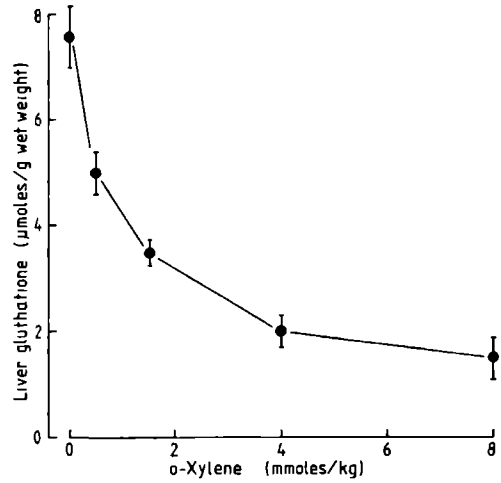

Fig. 1. Effect of o-xylene on the hepatic glutathione concentration as a function of the dose. Concentrations were determined 3 h after the administration of o-xylene (2.00 p.m.). Values are means (± SEM) of three rats

Table 2. Incubation of o-xylene with glutathione in the presence of 105,000 *g* supernatant of rat liver homogenate; effect on the glutathione concentration in the incubation medium

o-Xylene concentration (in mMolar)	Glutathione concentration[a]	
	Heat-denatured supernatant[b]	Supernatant
0	1367 ± 5	1408 ± 4
0.5	1364 ± 18	1399 ± 12
1.0	1363 ± 2	1411 ± 21
2.0	1362 ± 15	1409 ± 6

[a] Glutathione concentrations were expressed in 10^{-6} M (mean values ± SEM of three determinations)
[b] 10 min at 100° C

(2) enzymatic conjugation mediated by one of the GSH S-transferases; (3) bioactivation by cellular enzymes, followed by enzymatic or non-enzymatic conjugation of a reactive metabolite with GSH.

Table 2 illustrates that direct conjugation of o-xylene with GSH does not occur in vitro, neither spontaneously nor in the presence of 105,000 *g* supernatant of rat liver homogenate containing the GSH S-transferases. It is concluded, therefore, that o-xylene is bio-activated by other cellular enzymes before it reacts with GSH.

Increase in Urinary Thioether Concentration

Conjugation products of xenobiotic compounds with GSH are often excreted as mercapturic acids or cysteine conjugates in urine. Accordingly, the excretion of thioethers in urine was determined 24 and 48 h after treatment of the rats with toluene and xylenes. The results are shown in Table 3. Administration of toluene and xylenes

Table 3. The excretion of toluene and xylenes as thioethers in the rat

Treatment[a]	Route	Dose (mmoles/kg)	Urinary thioether excretion[b]		% of the dose[c]
			First day	Second day	
Control		–	6 ± 2	7 ± 2	–
Toluene	oral	1.0	17 ± 1	7 ± 1	0.5
		2.0	20 ± 3	8 ± 1	0.4
		3.0	26 ± 3	9 ± 3	0.4
		4.0	37 ± 3	8 ± 3	0.5
	i.p.	1.0	21 ± 2	7 ± 1	0.7
		2.0	26 ± 3	7 ± 2	0.5
		3.0	31 ± 4	10 ± 1	0.5
		4.0	47 ± 8	10 ± 2	0.5
o-Xylene	i.p.	0.5	180 ± 20	10 ± 2	21
		1.0	270 ± 20	17 ± 4	16
		1.5	330 ± 30	34 ± 4	14
		3.0	420 ± 30	96 ± 5	10
m-Xylene	i.p.	3.0	69 ± 4	11 ± 1	1.3
p-Xylene	i.p.	3.0	34 ± 2	10 ± 1	0.6

[a] The compounds were administered in 0.5 ml arachis oil

[b] Urinary thioether concentration is expressed as mmoles –SH/mole creatinine, using N-acetyl-L-cysteine as a standard in the SH-determination. Mean values ± SEM are presented ($n = 3$)

[c] The percentage of the dose excreted as mercapturic acid was calculated using N-acetyl-S-benzyl-L-cysteine as a reference for toluene and N-acetyl-S-(o-methyl-benzyl)-L-cysteine as a reference for the xylenes (see Fig. 4)

caused dose-dependent increases in the urinary thioether concentration. The enhanced excretion was almost completed within 24 h after administration of the compounds. The increase was most pronounced after treatment with o-xylene, the compound that was also most effective in hepatic GSH depletion.

Identification of Urinary Thioethers

Urine of ten rats was collected for 24 h after injection of o-xylene (200 mg/kg, i.p.). The urine samples were pooled, acidified to pH 2.0 with 4 N HCl, and extracted twice with two volumes of ethyl acetate. The combined ethyl acetate layers were evaporated to dryness and the residue was mixed vigourously by sonication in diethyl ether. After centrifugation at 3000 *g* for 5 min the solvent was separated and removed in vacuo. The residue was washed several times with hexane. A small volume of diethyl ether was added and the crystallizing compound was re-crystallized in hexane/ethanol. From the resulting white crystals mass- and NMR-spectra were obtained and the m.p. was determined.

The white crystalline compound was shown to be identical to N-acetyl-S-(o-methylbenzyl)-L-cysteine (Table 4, Fig. 2). The m.p. of the isolated compound was the same as the m.p. of the synthetized N-acetyl-S-(o-methylbenzyl)-L-cysteine. Hence, it

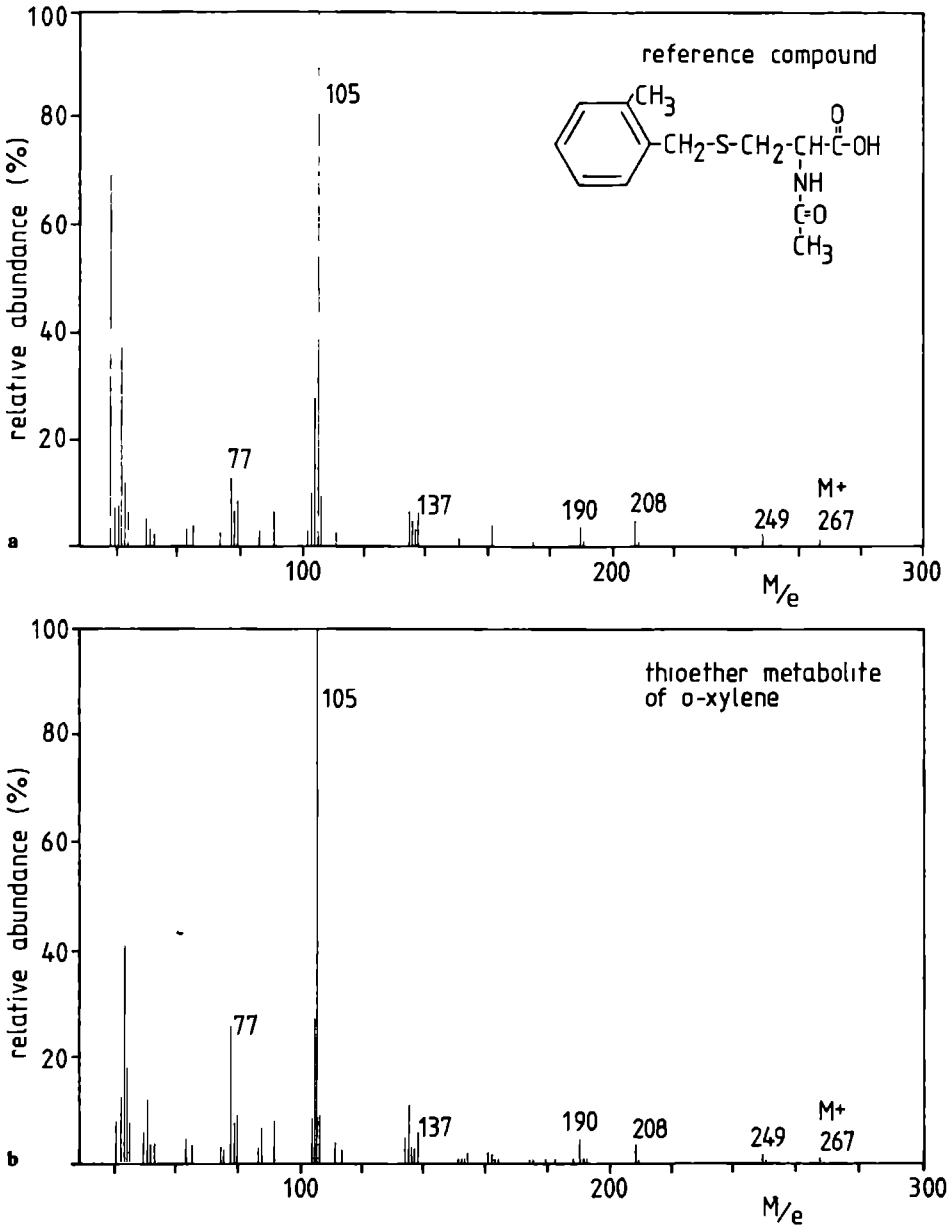

Fig. 2a and b. Mass spectra of thioethers. (**a**) The reference compound N-acetyl S-(o-methylbenzyl)-L-cysteine; (**b**) thioether metabolite isolated from urine of rats after administration of o xylene

is concluded that a mercapturic acid that originates from side-chain metabolism, appears in the urine of rats dosed with o-xylene.

Urine from four rats treated with toluene (4.0 mmoles/kg, i.p.) was collected for 24 h, pooled and subjected to thin layer chromatography as described in the "Materials and Methods" section. The Rf-values of a thioether compound that was present in urine

Table 4. NMR data of a thioether isolated from the urine of rats treated with o-xylene and of a synthetic mercapturic acid

Assignment	Integral	Synthetic N-acetyl-S-(o-methylbenzyl)cysteine (in $CDCl_3$) (ppm)[a]	Isolated thioether (in CD_3OD)[b] (ppm)[a]
$-COC\underline{H}_3$	3	2.01 (s)	2.00 (s)
$-C_6H_4-C\underline{H}_3$	3	2.37 (s)	2.38 (s)
$-S-C\underline{H}_2-$	2	2.95–3.01 (m)	2.87–3.03 (m)
$-C_6H_4-C\underline{H}_2-$	2	3.73 (s)	3.78 (s)
$-CH_2-C\underline{H}-$	1	4.78–4.85 (m)	4.71–4.85 (m)
$=CH-N\underline{H}-$	1[c]	6.40 (d)	exchanges with solvent
$-C_6\underline{H}_4-$	4	7.16 (s)	7.14–7.16 (m)

[a] NMR spectra were made with TMS at 0.00 ppm
[b] CD_3OD; solvent signals at 3.32 and 4.85 ppm
[c] No signal in CD_3OD
Abbreviations: (s) singlet; (d) doublet, and (m) multiplet

of toluene treated rats were found to be equal to the Rf-values of N-acetyl-S-benzyl-L-cysteine in different solvent systems (Fig. 3). The Rf-values were calculated to be 0.35, 0.69, and 0.63 with the solvent systems (a), (b), and (c), respectively. This indicates that benzylmercapturic acid appeared in rat urine as a metabolite of toluene.

Quantification of Mercapturic Acid Excretion

The reference compounds S-benzyl-L-cysteine, N-acetyl-S-benzyl-L-cysteine and N-acetyl-S-(o-methylbenzyl)-L-cysteine were added to rat urine to reach concentrations ranging from 0.06 to 10.0 mM. According to the procedure described in the "Materials and Methods" section thioether-values were determined and plotted against the urinary concentration of the reference compounds (Fig. 4). A very low recovery (0.7%) was found for the cysteine conjugate as compared with those of the N-acetyl-cysteine conjugates (67 and 71%). Thus, the assay procedure is far more selective for mercapturates than for cysteine conjugates.

As is shown in Table 3, it was calculated that only minor amounts of toluene, m- and p-xylene were excreted as mercapturic acids in urine. o-Xylene, however, was excreted as mercapturic acid for 10–21% of the dose.

Discussion

The results described here demonstrate that administration of toluene and xylenes to rats causes a decreased GSH concentration in the liver. This effect was most pronounced after administration of o-xylene (Table 1). The decreases in GSH concentration after various doses of o-xylene are comparable with the degree of depletion found after administration of bromobenzene to rats (Jollow et al., 1974).

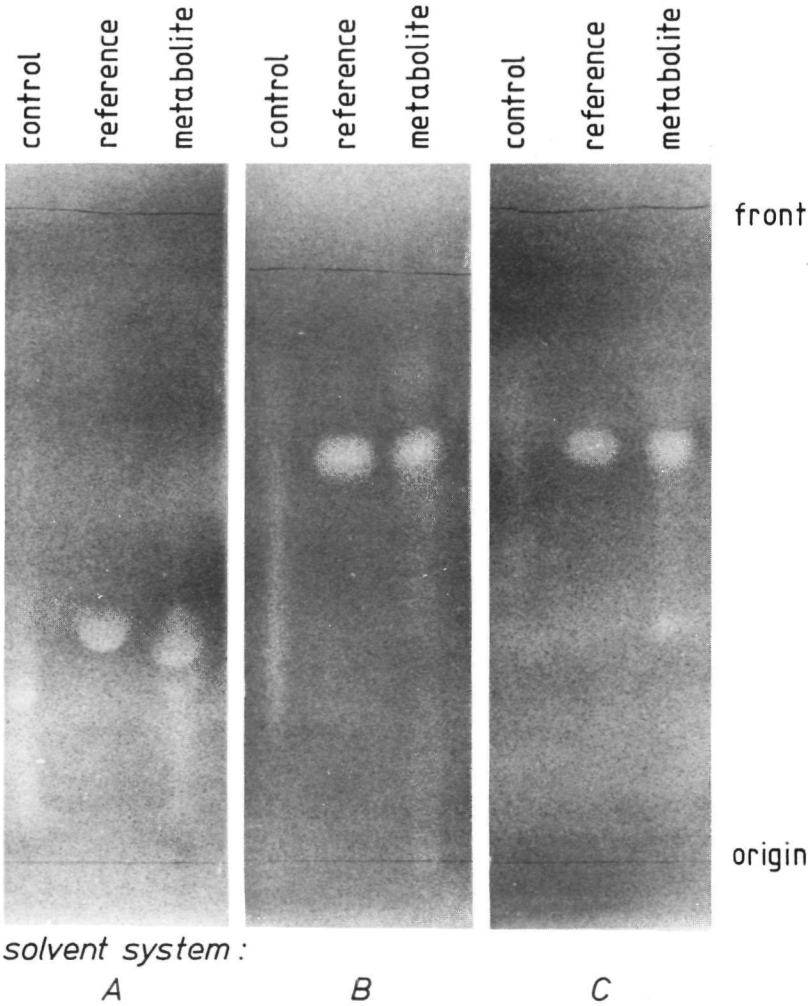

Fig. 3. Thin layer chromatography of rat urine extracts. Solvent systems: **A**: butanol-water-ammonia (25%) (3 : 1 : 1, v/v); **B**: methylene chloride-methanol-acetic acid (10 : 4 : 1, v/v); **C**: butanol-water-acetic acid (3 : 1 : 1, v/v). Control refers to a urine extract obtained from rats given arachis oil only. Metabolite refers to an extract obtained from urine of rats treated with toluene (4.0 mmoles/kg), in arachis oil. The reference compound was benzylmercapturic acid

The GSH depletion was accompanied by an enhanced excretion of thioether compounds in the urine. The thioether excretion was highest after treatment of the animals with o-xylene. From the in vitro experiments on the conjugation of o-xylene with GSH (Table 2), it is evident that not o-xylene itself but a metabolite of o-xylene reacts with GSH.

It has been reported that phenolic metabolites appear in the urine of rats exposed to toluene and xylenes (Bakke and Scheline, 1970), and that small amounts of o-cresol, which is not a normal constituent of urine, are found in urine of printing workers

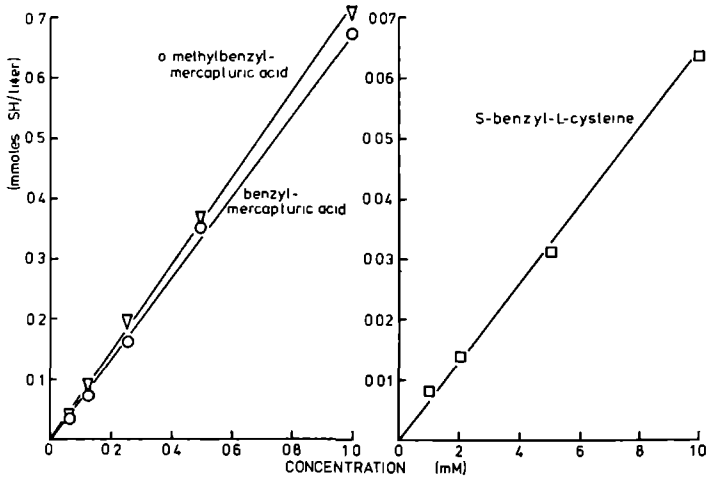

Fig. 4. Recovery of o-methylbenzyl mercapturic acid, benzylmercapturic acid and S-benzyl-L-cysteine in the thioether determination. The compounds were solubilized in rat urine and subjected to the procedure described under "Determination of Urinary Thioether Excretion". The sulfhydryl concentration found was corrected for the attribution of thiols and thioethers normally present in rat urine

exposed to toluene (Angerer, 1979). These findings suggest that aromatic epoxides are minor intermediary metabolites of toluene and xylenes. Therefore, one might suppose that the decrease in liver GSH and the concomittant thioether excretion in urine is due to detoxication of aromatic epoxides.

However, the thioether compound that was excreted in the urine of rats treated with o-xylene, was identified as o-methylbenzyl mercapturic acid by means of NMR and GC-MS (Table 4, Fig. 2). This finding, and in addition, the chromatographic evidence for the presence of benzylmercapturic acid in the urine of rats treated with toluene, strongly suggest that an intermediate resulting from side-chain metabolism is involved in the depletion of liver GSH.

Although side-chain metabolism leading to the urinary excretion of mercapturic acids appears to be a minor pathway in the biotransformation of toluene, m- and p-xylene, a substantial fraction of o-xylene (10—20% of the dose) is eliminated as o-methylbenzyl mercapturic acid. Bray et al. (1949), who studied the metabolism of the three xylenes in rabbits, found that about 60% of o-xylene, and 81 and 88% respectively of m- and p-xylene were converted into toluic acids and their glycine conjugates. Our results showing that 10—20% of o-xylene is converted into mercapturic acid may explain this difference and clarify a thusfar unknown metabolic pathway of toluene and the xylenes.

The formation of glutathione conjugates and the excretion of mercapturic acids can generally be considered as the result of detoxication of electrophiles in the organism (Arias and Jakoby, 1976; Chasseaud, 1979). Hence it may be doubted whether side-chain oxidation is a warrant of "safe metabolism" in the biotransformation of methyl-substituted benzenes.

A decade ago it was shown that benzyl and menaphthyl esters afford mercapturic acids in the rat in vivo and that sulphate esters are converted in a relatively high yield

(Hyde and Young, 1968; Clapp and Young, 1970; Gillham et al., 1970). Menaphthyl and benzyl sulphate are substrates for a glutathione S-transferase, which converts these esters to thioethers (Gillham, 1971, 1973). Menaphthyl alcohol is not a substrate. The metabolism of toluene and xylenes to mercapturic acids might therefore involve sequential oxidation, sulphation and glutathione conjugation.

Acknowledgements. We wish to thank Dr. H. C. J. Ketelaars and Mr. H. H. K. Brinkhof for their help in the performance and interpretation of the mass- and NMR-spectra, respectively. The advice of Dr. L. P. C. Delbressine in the synthesis of the reference compounds is also gratefully acknowledged.

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NON-MUTAGENICITY OF TOLUENE, *o*-, *m*- AND *p*-XYLENE, *o*-METHYLBENZYLALCOHOL AND *o*-METHYLBENZYLSULFATE IN THE AMES ASSAY

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Summary

Toluene, *o*-, *m*- and *p*-xylene, *o*-methylbenzylalcohol and *o*-methylbenzylsulfate were assayed for mutagenicity in the Ames assay. These compounds were unable to revert *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA98 and TA100, either with or without metabolic activation by S9 mix derived from livers of rats either untreated or induced with Aroclor 1254.

Toluene and xylenes are major constituents of coal-tar naphtha and crude oil. Their use as solvents is very common. Recently we found that administration of toluene and xylenes to rats caused a decrease in the concentration of liver glutathione (van Doorn et al., 1980a). The effect was most pronounced after the administration of *o*-xylene. A metabolite of this compound reacts with glutathione. These findings suggest that, during the metabolism of toluene and xylenes, alkylating intermediates are formed which react with glutathione. We now have circumstantial evidence that the metabolism of *o*-xylene leading to the formation of the glutathione conjugate proceeds via side-chain oxidation and sulfation (van Doorn et al., 1980b).

It is an important question whether, owing to their potential alkylating properties, one or more of the metabolic products of these methylated benzene derivatives are mutagenic. In the present study we tested toluene, *o*-, *m*- and *p*-xylene, *o*-methylbenzylalcohol and *o*-methylbenzylsulfate for mutagenicity in the Salmonella/microsome assay described by Ames et al. (1975).

Materials and methods

Chemicals

Citric acid monohydrate and *o*-, *m*- and *p*-xylene were obtained from J.T.

TABLE 1

RESULTS OF THE AMES ASSAY ON TOLUENE DERIVATIVES USING 5 *Salmonella typhimurium* STRAINS WITHOUT ACTIVATION

Compound tested	Amount μg/plate	Number of his$^+$ revertants per plate [a]				
		Strain				
		TA1535	TA1537	TA1538	TA98	TA100
Toluene	0	9 ± 2	4 ± 2	18 ± 1	25 ± 5	70 ± 8
	100	5 ± 2	3 ± 1	21 ± 2	18 ± 3	83 ± 4
	200	6 ± 1	5 ± 1	26 ± 8	21 ± 2	73 ± 8
	500	11 ± 4	4 ± 1	23 ± 3	21 ± 4	65 ± 4
	1000	10 ± 3	4 ± 1	25 ± 4	19 ± 1	56 ± 5
	2000	8 ± 2	6 ± 1	19 ± 3	22 ± 1	72 ± 10
o-Xylene	20	9 ± 2	8 ± 1	18 ± 3	29 ± 3	78 ± 7
	50	8 ± 2	8 ± 3	19 ± 3	23 ± 2	80 ± 12
	100	8 ± 1	4 ± 2	20 ± 1	25 ± 3	71 ± 5
	200	7 ± 3	4 ± 1	19 ± 3	21 ± 4	79 ± 5
	500	7 ± 4	5 ± 1	19 ± 4	19 ± 3	70 ± 5
m-Xylene	20	6 ± 1	5 ± 1	23 ± 3	26 ± 3	69 ± 4
	50	9 ± 2	5 ± 2	21 ± 2	28 ± 1	70 ± 2
	100	7 ± 1	5 ± 2	21 ± 2	21 ± 4	84 ± 10
	200	9 ± 2	6 ± 1	25 ± 5	28 ± 6	73 ± 4
	500	7 ± 1	4 ± 1	28 ± 2	20 ± 3	71 ± 5

p-Xylene	20	6 ± 1	4 ± 2	20 ± 2	24 ± 2	65 ± 9
	50	10 ± 2	4 ± 1	18 ± 2	23 ± 5	63 ± 4
	100	12 ± 2	4 ± 1	24 ± 2	20 ± 3	62 ± 5
	200	9 ± 2	4 ± 1	15 ± 1	26 ± 1	78 ± 6
	500	10 ± 2	6 ± 1	21 ± 5	25 ± 5	76 ± 8
o-Methylbenzyl alcohol	100	7 ± 3	6 ± 2	27 ± 3	25 ± 1	93 ± 3
	200	10 ± 2	6 ± 1	21 ± 2	22 ± 3	72 ± 5
	500	11 ± 2	4 ± 1	22 ± 2	26 ± 3	65 ± 8
	1000	8 ± 1	3 ± 1	22 ± 3	23 ± 2	64 ± 1
	2000	9 ± 2	4 ± 1	20 ± 1	20 ± 4	61 ± 1
o-Methylbenzyl sulfate	0 [b]	7 ± 1	10 ± 2	20 ± 3	26 ± 1	98 ± 4
	100	7 ± 3	6 ± 1	19 ± 3	23 ± 2	91 ± 14
	200	8 ± 2	4 ± 1	19 ± 4	25 ± 2	85 ± 4
	500	9 ± 2	6 ± 3	24 ± 4	27 ± 1	93 ± 11
	1000	6 ± 1	7 ± 4	20 ± 3	21 ± 3	82 ± 14
	2000	9 ± 2	8 ± 2	26 ± 2	24 ± 2	100 ± 7
Reference mutagen						
Sodiumazide	5	2100 ± 100	6 ± 1	24 ± 2	26 ± 8	2400 ± 200
4-Nitroquinoline-1-oxide	2	35 ± 3	14 ± 4	175 ± 7	417 ± 12	4100 ± 120
4-Nitro-*o*-phenylenediamine	10	17 ± 1	42 ± 1	1760 ± 36	1350 ± 83	284 ± 23
9-Aminoacridine	200	7 ± 1	8900 ± 200	10 ± 2	18 ± 3	46 ± 16
Spontaneous		11 ± 3	7 ± 1	24 ± 2	37 ± 3	100 ± 5

[a] Mean values ± S.E.M. of determinations in triplicate.

[b] Unlike the other compounds, which were dissolved in DMSO, *o*-methylbenzyl sulfate was given in aqueous solution.

Baker Chemicals (Deventer, The Netherlands). *o*-Methylbenzylalcohol, 9-aminoacridine hydrochloride monohydrate, 2-aminoanthracene, benzo[*a*]pyrene, 4-nitro-*o*-phenylenediamine and 4-nitroquinoline-1-oxide were purchased from Aldrich Europe (Beerse, Belgium). Sodium azide and toluene were purchased from Merck (Darmstadt, FRG), D-biotin and L-histidine—HCl from Sigma (St.

TABLE 2

RESULTS OF THE AMES ASSAY ON TOLUENE DERIVATIVES

5 *Salmonella typhimurium* strains were used with activation by S9 mix from livers of untreated rats.

Compound tested	Amount μg/plate	Number of his$^+$ revertants per plate [a]				
		Strain				
		TA1535	TA1537	TA1538	TA98	TA100
Toluene	0	8 ± 3	5 ± 1	22 ± 5	25 ± 1	88 ± 9
	100	13 ± 3	5 ± 1	19 ± 2	13 ± 3	98 ± 4
	200	14 ± 3	4 ± 2	14 ± 3	31 ± 2	79 ± 3
	500	10 ± 3	3 ± 1	24 ± 1	29 ± 3	81 ± 6
	1000	12 ± 1	6 ± 1	17 ± 2	34 ± 1	83 ± 6
	2000	11 ± 1	5 ± 2	19 ± 1	35 ± 3	74 ± 6
o-Xylene	20	14 ± 1	4 ± 1	19 ± 3	32 ± 4	73 ± 9
	50	7 ± 1	5 ± 2	11 ± 1	26 ± 6	80 ± 8
	100	7 ± 2	3 ± 1	16 ± 1	14 ± 2	89 ± 18
	200	12 ± 3	4 ± 1	13 ± 2	26 ± 2	82 ± 1
	500	6 ± 1	9 ± 4	19 ± 5	29 ± 1	78 ± 7
m-Xylene	20	13 ± 1	9 ± 2	16 ± 2	33 ± 3	84 ± 3
	50	10 ± 3	6 ± 1	17 ± 1	24 ± 3	68 ± 4
	100	8 ± 2	5 ± 1	23 ± 4	26 ± 3	78 ± 2
	200	8 ± 2	8 ± 3	18 ± 2	29 ± 1	80 ± 2
	500	8 ± 3	5 ± 1	20 ± 3	24 ± 5	101 ± 6
p-Xylene	20	11 ± 2	4 ± 1	17 ± 4	36 ± 6	90 ± 10
	50	12 ± 3	4 ± 1	17 ± 2	30 ± 1	88 ± 2
	100	10 ± 3	5 ± 2	20 ± 3	30 ± 5	75 ± 5
	200	9 ± 1	5 ± 2	16 ± 2	29 ± 2	78 ± 6
	500	6 ± 1	4 ± 1	26 ± 6	38 ± 8	88 ± 13
o-Methylbenzyl alcohol	100	11 ± 3	6 ± 1	21 ± 3	15 ± 1	82 ± 7
	200	8 ± 2	5 ± 3	20 ± 2	17 ± 1	81 ± 4
	500	14 ± 1	5 ± 1	24 ± 2	22 ± 4	83 ± 6
	1000	14 ± 1	7 ± 4	19 ± 4	25 ± 1	66 ± 3
	2000	11 ± 1	3 ± 1	17 ± 1	29 ± 3	88 ± 5
o-Methylbenzyl sulfate	0 [b]	10 ± 2	6 ± 1	20 ± 1	27 ± 2	74 ± 2
	100	10 ± 2	4 ± 1	14 ± 1	21 ± 4	90 ± 8
	200	6 ± 1	5 ± 1	22 ± 3	16 ± 3	83 ± 10
	500	12 ± 1	5 ± 2	24 ± 5	20 ± 4	79 ± 8
	1000	16 ± 2	5 ± 0	26 ± 8	26 ± 3	69 ± 5
	2000	7 ± 1	10 ± 3	18 ± 2	30 ± 2	82 ± 3
Reference mutagen						
2-Aminoanthracene	1	794 ± 28	175 ± 6	1685 ± 57	1345 ± 71	7400 ± 250
Benzo[*a*]pyrene	7.5	25 ± 9	5 ± 2	39 ± 3	—	339 ± 17
9,10-Dimethylbenzanthracene	20	13 ± 1	138 ± 6	255 ± 12	68 ± 3	183 ± 9
Spontaneous		8 ± 3	5 ± 2	20 ± 3	22 ± 2	91 ± 3

[a] Mean values ± S.E.M. of determinations in triplicate.

[b] Unlike the other compounds, which were all dissolved in DMSO, *o*-methylbenzyl sulfate was given in aqueous solution.

Louis, U.S.A.). Nicotinamide adenine dinucleotide phosphate (NADP) disodium salt and glucose 6-phosphate (G-6-P) disodium salt were purchased from Boehringer (Mannheim, F.R.G.). Purified agar was purchased from Difco Laboratories (Detroit, U.S.A.), 9,10-dimethyl-1,2-benzanthracene from ICN Pharmaceuticals and nutrient broth No. 2 from Oxoid Ltd (Basingstoke, England).

TABLE 3

RESULTS OF THE AMES ASSAY ON TOLUENE DERIVATIVES

5 *Salmonella typhimurium* strains were used with activation by S9 mix from livers of Aroclor-treated rats.

Compound tested	Amount μg/plate	Numbers of his$^+$ revertants per plate [a]				
		Strain				
		TA1535	TA1537	TA1538	TA98	TA100
Toluene	0	11 ± 4	7 ± 3	24 ± 5	24 ± 3	89 ± 15
	100	7 ± 1	8 ± 3	24 ± 2	32 ± 3	85 ± 6
	200	11 ± 4	9 ± 2	26 ± 7	26 ± 4	96 ± 9
	500	11 ± 2	10 ± 2	18 ± 1	26 ± 1	99 ± 5
	1000	8 ± 1	5 ± 2	21 ± 4	24 ± 3	83 ± 11
	2000	7 ± 1	7 ± 2	21 ± 4	28 ± 5	71 ± 3
o-Xylene	20	12 ± 4	13 ± 1	28 ± 1	28 ± 2	92 ± 3
	50	8 ± 1	10 ± 3	25 ± 1	24 ± 1	98 ± 8
	100	18 ± 3	9 ± 4	22 ± 4	33 ± 6	80 ± 6
	200	14 ± 4	7 ± 3	23 ± 5	21 ± 4	89 ± 7
	500	14 ± 1	7 ± 3	24 ± 4	31 ± 2	92 ± 6
m-Xylene	20	12 ± 4	9 ± 2	28 ± 3	32 ± 6	98 ± 6
	50	10 ± 3	11 ± 2	19 ± 1	31 ± 1	94 ± 9
	100	7 ± 1	5 ± 1	24 ± 3	26 ± 4	95 ± 4
	200	10 ± 1	7 ± 1	21 ± 3	29 ± 1	82 ± 4
	500	12 ± 1	8 ± 1	22 ± 1	29 ± 1	98 ± 7
p-Xylene	20	9 ± 2	11 ± 2	17 ± 2	29 ± 1	94 ± 13
	50	10 ± 2	12 ± 2	19 ± 2	27 ± 1	85 ± 7
	100	11 ± 4	9 ± 1	22 ± 2	26 ± 4	83 ± 5
	200	7 ± 1	8 ± 3	21 ± 4	32 ± 4	78 ± 6
	500	10 ± 3	10 ± 3	22 ± 3	24 ± 2	94 ± 5
o-Methylbenzyl alcohol	100	8 ± 3	8 ± 3	19 ± 1	24 ± 2	93 ± 4
	200	7 ± 1	7 ± 1	24 ± 1	26 ± 1	80 ± 7
	500	7 ± 2	4 ± 1	22 ± 1	31 ± 2	81 ± 2
	1000	12 ± 0	10 ± 2	17 ± 2	18 ± 1	84 ± 10
	2000	7 ± 1	6 ± 1	25 ± 4	35 ± 2	83 ± 5
o-Methylbenzyl sulfate	0 [b]	8 ± 3	8 ± 1	26 ± 8	31 ± 3	93 ± 2
	100	10 ± 3	5 ± 1	23 ± 2	27 ± 3	108 ± 2
	200	13 ± 1	7 ± 1	25 ± 5	23 ± 2	83 ± 8
	500	13 ± 3	10 ± 3	25 ± 2	26 ± 2	107 ± 5
	1000	11 ± 3	6 ± 1	21 ± 5	20 ± 2	105 ± 14
	2000	7 ± 1	11 ± 3	22 ± 2	28 ± 4	112 ± 10
Reference mutagen						
2-Aminoanthracene	1	132 ± 14	54 ± 5	538 ± 22	281 ± 8	650 ± 4
Benzo[*a*]pyrene	7.5	19 ± 11	222 ± 10	381 ± 14	1230 ± 60	870 ± 34
9,10-Dimethylbenzanthracene	20	16 ± 2	126 ± 3	57 ± 4	379 ± 11	491 ± 15
Spontaneous		8 ± 3	9 ± 1	20 ± 1	30 ± 2	104 ± 9

[a] Mean values ± S.E.M. of determinations in triplicate.

[b] Unlike the other compounds, which were dissolved in DMSO, *o*-methylbenzyl sulfate was given in aqueous solution.

o-Methylbenzylsulfate was synthesized from *o*-methylbenzylalcohol according to Clapp and Young (1970).

Mutagenicity testing

The mutagenicity test was performed according to Ames et al. (1975) with *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA98 and TA100. We used Oxoid nutrient broth instead of Difco nutrient broth.

Rat liver S9 (9000 *g* supernatant) fractions were prepared from male Wistar rats either untreated or pretreated with Aroclor 1254. S9 mix contained 0.1 ml S9 per ml.

Plates were counted after 48 h incubation at 37°C with a Biotran II automated colony counter.

Results

Table 1 shows that none of the tested toluene derivatives was directly mutagenic to the *Salmonella typhimurium* strains. The mutability of the strains was verified with the directly acting mutagens: sodium azide, 4-nitroquinoline-1-oxide, 4-nitro-*o*-phenylenediamine and 9-aminoacridine. Tables 2 and 3 show that none of the toluene derivatives was mutagenic to *Salmonella typhimurium* after activation with liver S9 mix derived from either untreated or Aroclor-1254-treated rats. To verify both mutability of the strains and efficiency of the metabolic activation system, we used the premutagens: 2-aminoanthracene, benzo[*a*]pyrene and 9,10-dimethyl-1,2-benzanthracene.

Killing of the bacteria was only found to a low extent; at the highest concentrations 80—100% of the bacteria survived.

Discussion

In a previous study we obtained evidence that, during the metabolism of toluene and xylenes, reactive intermediates are formed, probably due to side-chain oxidation and sulfation. This in particular holds for *o*-xylene (van Doorn et al., 1980b). No mutagenicity could be detected for toluene, *m*-xylene or *p*-xylene; and *o*-xylene, *o* methylbenzylalcohol and *o*-methylbenzylsulfate were also found negative. We also investigated, using the method described by Brouns et al. (1979), whether these compounds are able to evoke DNA-excision repair in suspensions of freshly isolated rat hepatocytes. All these compounds, at concentrations ranging from 10^{-7} to 10^{-3} M, were negative. Gerner-Smidt and Friedrich (1978) studied the genotoxic effect of benzene, toluene and xylene by the SCE technique. They did not find any increase, either in the frequency of SCEs or in the number of structural chromosomal aberrations. Data about genotoxic effects of toluene were gathered by Dean (1978). He has been unable to trace any reference in the literature to microbial mutation testing of toluene. Reported animal experiments show that high concentrations of toluene are required to induce chromosomal aberrations. No significant increase could be detected in chromosomal aberrations in workers exposed to concentrations of toluene close to the maximal allowable concentration of 200 ppm for periods up to 15 years.

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Chapter 3

The role of the activating system in bacterial mutagenicity testing

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Comparison of the mutagenicities of 4-aminobiphenyl and benzidine in the Salmonella/microsome, Salmonella/hepatocyte and host-mediated assays

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Summary

The applicability of suspensions of rat hepatocytes as the metabolic factor in a bacterial mutagenicity test was studied. Therefore, mutagenicity of 4-aminobiphenyl and benzidine towards the *Salmonella typhimurium* strains TA1538 and TA100 in the presence of rat-hepatic microsomal preparations or intact liver cells was measured. The mutagenic potency of these compounds was also established in the intrasanguineous host-mediated assay.

Comparison of the test results showed that the mutagenic activity of 4-aminobiphenyl and benzidine after metabolism by intact rat hepatocytes resembled the mutagenic activity of these compounds after biotransformation in vivo more than after activation with a rat-liver S9 fraction.

The Salmonella/microsome assay is widely used for the detection of mutagens [1]. Because many mutagens need metabolic conversion to generate their genotoxic properties, in this test in vitro mutagenic activation is mediated by the addition of a rat-liver S9 fraction. It is uncertain, however, to what extent the mutagenic activation by the enzymes present in the S9 fraction approximates the biotransformation in vivo, because during preparation of the S9 fraction, activating and deactivating enzymes are dissociated [5]. It may also be doubted whether the concentration of co-factors applied in the test in vitro reflects the situation in vivo [5]. For instance, the S9 fraction does not contain co-factors for the acetylation [10]. In general, the S9 fraction catalyses phase-I reactions and contains little activity of phase-II reactions [8]. On the one hand, this could be the reason that the Salmonella/microsome assay

is inadequate for some classes of mutagen [5]. On the other hand, it could be possible to detect mutagens that are not detectable after activation in vivo.

In the intrasanguineous host-mediated assay the indicator cells, in this case Salmonella bacteria, are injected into a lateral tail vein and are recovered mainly from the liver to be screened for the presence of mutants after treatment of the animals with a suspected compound [9,12]. Although the compound under test undergoes metabolism in vivo, this test system suffers from insensitivity. Probably because, in vivo, the systems are protected by urinary, biliary and respiratory excretion systems, high dose levels to the host are needed of certain compounds [5]. However, these levels are limited by the toxicity of the compounds to the host [11]. Because this system is biased to false negative responses, it should not be used as a primary mutagenic screen [11].

Because, in the total animal, metabolic activation is achieved mainly in the liver, we think that isolated rat hepatocytes deserve serious attention as a tool in mutagenicity studies. Mutagenic activation by intact hepatocytes may give a better approximation of the situation in vivo than does the rat-liver S9 fraction. Moreover, disturbing effects that occur in total animals owing to test compounds showing acute toxicity are eliminated.

In this study we compared the mutagenicity of 2 structurally related carcinogenic aryl amines, 4-aminobiphenyl and benzidine, which are subject to a rather complex metabolism in vivo, in 3 different test methods: the Salmonella/microsome, the Salmonella/hepatocyte and the intrasanguineous host-mediated assays.

Materials and methods

Chemicals

Benzidine was purchased from Merck (Darmstadt, F.R.G.). 4-Aminobiphenyl was from Aldrich Europe (Beerse, Belgium). D-Biotin, L-histidine-HCl and collagenase type I were obtained from Sigma (St. Louis, U.S.A.). Nicotinamide adenine dinucleotide phosphate (NADP) disodium salt and glucose-6-phosphate (G-6-P) disodium salt were purchased from Boehringer (Mannheim, F.R.G.). Purified agar was from Difco Laboratories, nutrient broth No. 2 from Oxoid Ltd. (Basingstoke, England) and citric acid monohydrate from J.T. Baker Chemicals (Deventer, The Netherlands).

Preparation of isolated hepatocytes

The procedure was based on the methods described by Berry and Friend [3] and by Seglen [14], except for some modifications. All manipulations were performed under sterile conditions. Before the procedure, rats were anaesthetized by an i.p. injection of sodium pentobarbital (60 mg/ml distilled water, 0.35 ml). After 15 min of pre-perfusion of the liver with a Ca^{2+}-free HEPES buffer, this buffer was replaced by a collagenase-containing HEPES buffer (0.06% w/v collagenase) for another 15 min. The perfusion rate was 25 ml/min. The crude cell suspension was treated according to the method of Seglen [14], and the final pellet was resuspended

in a Ca^{2+}-containing HEPES–TES buffer solution and diluted to a density of 5×10^6 cells/ml. Trypan-blue exclusion showed the presence of about 95% viable cells.

Salmonella/microsome mutagenicity assay

The Salmonella/microsome assay was performed according to Ames et al. [1] with the *Salmonella typhimurium* strains TA1538 and TA100. We used Oxoid nutrient broth instead of Difco nutrient broth. Rat-liver S9 (9000 *g* supernatant) fractions were prepared from male Wistar rats either untreated or pretreated with phenobarbital. S9 mix contained 0.1 ml S9 per ml.

Salmonella intrasanguineous assay

The intrasanguineous assay was based on a method described by Mohn and Ellenberger [12]. Male Wistar rats weighing about 200 g were injected in the lateral tail vein with 1 ml of a saline suspension of *Salmonella typhimurium*. This suspension was prepared in the following way. 30 ml of an overnight-grown bacterial culture was inoculated into 100 ml of fresh nutrient broth and incubated in a shaker at 37°C. After 2.5 h, the bacterial suspension was centrifuged at 5000 *g*, and the pellet was resuspended in 1 ml of saline. A diluted suspension was plated for the estimation of the number of bacteria present in the suspension. According to the method described by Bakshi and Brusick [2], 45 min after bacterial injection, each animal was injected intraperitoneally with 4-aminobiphenyl or benzidine dissolved in sterile tragacanth (2%) at a dose of 1.0 mmole/kg. The control animals were injected with the same amount of sterile tragacanth. 1 h after the administration of the test compound, the rats were anaesthetized by an i.p. injection of sodium pentobarbital (60 mg/ml distilled water, 0.35 ml) and the livers were removed aseptically. They were minced with scissors and suspended in 40 ml of sterile saline. This suspension was homogenized and centrifuged at 200 *g*. The supernatant was centrifuged twice for 20 min at 1200 *g*, then the ultimate pellet was suspended in 1.0 ml of saline. Of this suspension, 0.1 ml was plated in triplicate with a molten top agar containing only biotin (ño histidine) for the determination of the number of *his*$^+$ revertants. Another 0.1 ml of the suspension was diluted 10^{-5}. Of this suspension, 0.1 ml was plated in triplicate with a molten top agar containing extra histidine for the determination of the total number of bacteria recovered. After incubation for 48 h at 37°C the number of colonies was counted, and the mutation frequency was calculated as the number of *his*$^+$ revertants/10^8 bacteria recovered.

Salmonella/hepatocyte mutagenicity assay

The procedure of the Salmonella/hepatocyte assay was carried out as follows. In a shaking water-bath, closed sterile vials, each containing 3.1 ml of hepatocyte suspension (5×10^6 viable cells/ml), 0.8 ml of an overnight-grown suspension of *Salmonella typhimurium* (about 1.5×10^9 bact./ml) and 0.1 ml of a 4-aminobiphenyl or benzidine solution (in DMSO) were incubated at 37°C for 1 h. Next, 0.5 ml of this mixture was plated in triplicate with a molten top agar containing only biotin (no histidine). The number of revertant colonies was counted after 48 h at 37°C.

Results

The mutagenicity of 4-aminobiphenyl and benzidine in the Salmonella/microsome assay

In Fig. 1 the mutagenicities of 4-aminobiphenyl and benzidine are shown towards the *Salmonella typhimurium* strains TA1538 and TA100 in the Salmonella/microsome assay in the presence of S9 mix. 4-Aminobiphenyl was slightly more mutagenic than benzidine towards strain TA1538. The mutagenicity of 4-aminobiphenyl was very high towards the tester strain TA100 as compared with benzidine (Fig. 1c, d). The mutagenic activity of both 4-aminobiphenyl and benzidine was increased when 9000 *g* supernatant of livers derived from phenobarbital-treated rats was used. Phenobarbital pretreatment strongly influenced the mutagenicity of 4-aminobiphenyl towards the tester strain TA100.

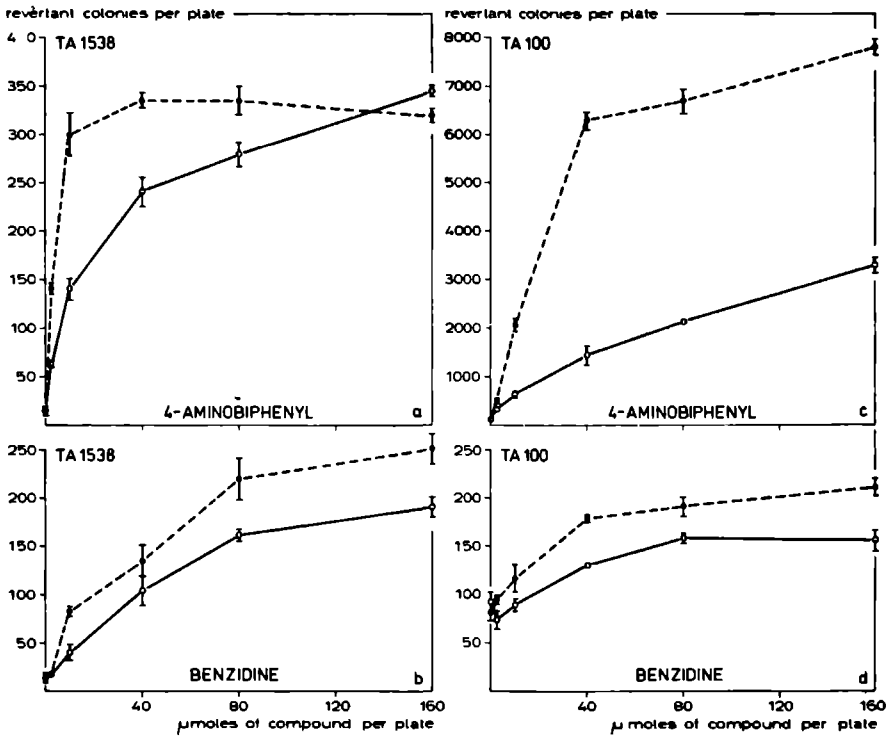

Fig 1 The mutagenicity of 4-aminobiphenyl and benzidine towards the *Salmonella typhimurium* strains TA1538 and TA100 in the plate assay in the presence of S9 mix S9 fractions were derived from untreated (————) or phenobarbital-pretreated rats (------) Mean values±S E M of determinations in triplicate

Mutagenicity of 4-aminobiphenyl and benzidine in the intrasanguineous host-mediated assay

The results of the host-mediated experiments are shown in Table 1. The recovery of the bacteria of both strains was low (0.4–2.4%).

The mutagenicities of the compounds under test are given as reversions per 10^8 survivors. After treatment of the rat with benzidine, the number of reversions per 10^8 survivors of strain TA1538 was very high in comparison with the number of reversions in the control rat. There was no significant increase in reversion rate after 4-aminobiphenyl treatment with respect to the control treatment. Pretreatment of

TABLE 1

MUTAGENIC ACTIVITY OF 4-AMINOBIPHENYL AND BENZIDINE IN THE HOST-MEDIATED ASSAY

Compound	Phenobarbital [a] pretreatment	Tester strain	Recovery [b] (%)	Reversions per 10^8 survivors
Control	–	TA1538	1.5	20
Control	–	TA1538	0.9	43
4-Aminobiphenyl	–	TA1538	0.8	37
4-Aminobiphenyl	–	TA1538	2.4	50
Benzidine	–	TA1538	0.5	272
Benzidine	–	TA1538	1.8	377
Control	+	TA1538	0.8	16
Control	+	TA1538	1.0	42
4-Aminobiphenyl	+	TA1538	0.9	118
4-Aminobiphenyl	+	TA1538	1.2	104
Benzidine	+	TA1538	1.2	323
Benzidine	+	TA1538	1.6	267
Control	–	TA100	0.5	93
Control	–	TA100	0.4	287
4-Aminobiphenyl	–	TA100	0.5	111
4-Aminobiphenyl	–	TA100	0.8	174
Benzidine	–	TA100	0.8	109
Benzidine	–	TA100	0.8	95
Control	+	TA100	0.2	217
Control	+	TA100	1.0	127
4-Aminobiphenyl	+	TA100	0.6	92
4-Aminobiphenyl	+	TA100	1.4	102
Benzidine	+	TA100	0.4	139
Benzidine	+	TA100	1.3	153

[a] Rats received 75 mg/kg per day for 4 days before the experiment

[b] Number of bacteria recovered from the liver as a fraction of the number injected (i.v.)

the rats with phenobarbital did not result in a significant difference in benzidine mutagenicity for TA1538. Treatment with 4-aminobiphenyl, however, led to an increase of the reversion rate.

With the *Salmonella typhimurium* strain TA100, no increase of the reversion rate was detected after treatment of the rats, either with 4-aminobiphenyl, or with benzidine. Pretreatment of the rats with phenobarbital had no detectable influence on the reversion rates.

The mutagenicity of 4-aminobiphenyl and benzidine in the Salmonella/hepatocyte assay

In Fig. 2 the mutagenicities of 4-aminobiphenyl and benzidine are shown after metabolic activation by intact rat-liver cells towards the *Salmonella typhimurium* strains TA1538 and TA100.

Benzidine was far more mutagenic than 4-aminobiphenyl when the tester strain

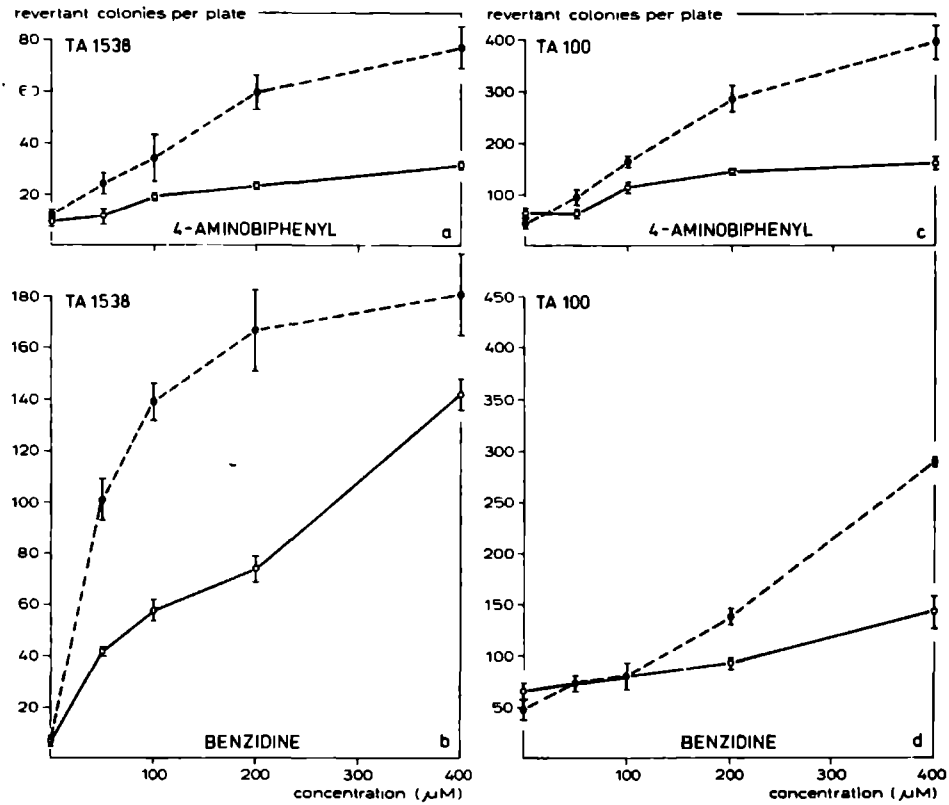

Fig 2 The mutagenicity of 4-aminobiphenyl and benzidine towards the *Salmonella typhimurium* strains TA1538 and TA100 after metabolic activation by rat-liver cells derived from untreated (————) or phenobarbital-pretreated rats (- - - - -) Experimental details were as mentioned in Materials and Methods Mean values ± S E M of 3 separate experiments

TA1538 was used and when metabolic activation was achieved by liver cells from untreated rats. When liver cells were used from phenobarbital-pretreated rats, we found a substantial increase in mutagenicity of both compounds, with benzidine still as the more active compound.

The mutagenicity of 4-aminobiphenyl towards the *Salmonella typhimurium* strain TA100 could hardly be measured after metabolic activation by rat-liver cells from untreated rats. However, when metabolic activation by rat-liver cells from phenobarbital-pretreated rats was achieved, the mutagenicity of 4-aminobiphenyl was well detectable to the *Salmonella typhimurium* strain TA100.

There was hardly any observable mutagenicity of benzidine to the *Salmonella typhimurium* strain TA100 when liver cells from untreated rats were used. Mutagenic activation to this strain was achieved to a low extent with liver cells that were isolated from phenobarbital-pretreated rats.

Discussion

Many mutagenic chemicals do not exert their genotoxic actions until they have been metabolized by the organism. Thereto, most mutagenicity tests in vitro, such as the frequently used Ames assay, must be supplied with an activating enzyme system, commonly a liver microsomal preparation. One has to realize that, in vivo, the mutagenic potency of the chemicals is determined by the balance between mutagenic activation and inactivation reactions.

Fig. 1 shows that, after activation with an S9 fraction, 4-aminobiphenyl was more mutagenic than benzidine. This particularly holds for the mutagenicity towards strain TA100. On the other hand, no mutagenicity of 4-aminobiphenyl was observed after metabolic activation in vivo (i.e. in the host-mediated assay, Table 1), whereas the mutagenicity of benzidine was well observable in this assay.

Considering the great discrepancy between the mutagenicity after incubation of benzidine and 4-aminobiphenyl in vitro or in vivo, it was interesting to compare the mutagenicity of 4-aminobiphenyl and benzidine with isolated intact hepatocytes as the metabolizing system (Fig. 2). The results are summarized in Table 2, which also shows the ratios between mutagenicity of 4-aminobiphenyl and benzidine, measured in the presence of various activating systems. These values are derived from the mutagenicity data on the highest concentrations tested. It is obvious that, with tester strain TA1538, benzidine was more mutagenic than 4-aminobiphenyl, both in the host-mediated assay and in the Salmonella/hepatocyte assay. This is in contrast with the results obtained by activation with the S9 liver fraction.

With tester strain TA100 we found no mutagenic activity of 4-aminobiphenyl or benzidine in the host-mediated assay and a low mutagenic response in the Salmonella/hepatocyte assay.

No marked changes in the ratios were observed after pretreatment of the rats with phenobarbital.

There is no doubt that the structural and functional organization of the enzymes involved in biotransformation is considerably disturbed in the rat-liver S9 fraction.

TABLE 2

MUTAGENICITY RATIO OF 4-AMINOBIPHENYL/BENZIDINE IN TA1538

Pretreatment	Activation system		
	S9 mix	Hepatocytes	Host-mediated
None	1 8	0 20	0 013
Phenobarbital	1 3	0 43	0 038
in TA100			
None	21	1 14	[a]
Phenobarbital	37	1 4	[a]

[a] 4-Aminobiphenyl and benzidine were both non-mutagenic towards TA100 in the host-mediated assay

Differences in cellular integrity may be the reason for a quantitative as well as qualitative difference between mutagenic metabolites formed in S9 mix and those released from hepatocytes both in vitro and in vivo.

The question arises whether the low sensitivity of the host-mediated assay, when compared with hepatocyte-mediated tests, is also due to the very low recovery of bacteria from rat livers and, therefore, to the low absolute numbers of induced *his* $^+$ mutant cells.

It is known that phenobarbital is capable of inducing several drug-metabolizing enzymes of the liver. Phenobarbital might influence in particular the activation of carcinogenic aryl amines, because these compounds are metabolized by a complex series of sequential steps to gain their toxic properties [16]. The increase in mutagenicity of 4-aminobiphenyl and benzidine after activation with a liver S9 fraction or with hepatocytes from phenobarbital-treated rats is in agreement with this. However, in the host-mediated assay, we only found a weak mutagenic response with 4-aminobiphenyl towards TA1538 after pretreatment of the rats with phenobarbital. This could mean that, for the dose applied, the capacity of the enzymes is not rate-limiting for the mutagenic activation in vivo. It was not possible to increase the dose because of the acute toxicity, particularly of 4-aminobiphenyl, to the rats.

Several investigators, including ourselves, have used isolated hepatocytes as a metabolic system in short-term mutagenicity testing [4,6,7,13,15]. From these studies it was suggested that the overall metabolism in the hepatocyte is fairly representative of the biotransformation in vivo. Polley et al. [13] concluded that intact hepatocytes may more efficiently produce mutagenic metabolites than do disrupted cell fractions. Other investigators found a better correlation between bacterial mutagenicity and carcinogenicity of benzo[*a*]pyrene and 4 of its major metabolites by using activation with intact liver cells instead of a cell homogenate [6].

The results of the present study show that, with the Salmonella/hepatocyte assay, mutagenic activities of 4-aminobiphenyl and benzidine are found that obviously differ from those measured in the Salmonella/microsome assay and resemble more closely the mutagenic activities observed with the host-mediated assay.

Acknowledgement

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Mutagenicity testing with the Salmonella/hepatocyte and the Salmonella/microsome assays

A comparative study with some known genotoxic compounds

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Summary

The applicability of isolated intact hepatocytes as a metabolic factor in bacterial mutagenicity screening was studied. Mutagenic activities of 12 known premutagenic compounds were determined in a *Salmonella typhimurium* test system comprising hepatocytes and were compared with mutagenicity data obtained with the commonly used Salmonella/microsome plate assay. In a qualitative sense the results obtained with the two systems were, in general, equivalent. However, some specific differences were found depending on the bacterial strain used. For instance, dimethylnitrosamine was only mutagenic for Salmonella strain TA1535 in the hepatocyte suspension system. On the other hand, benzo[*a*]pyrene was hardly mutagenic towards TA100 with hepatocytes in contrast with theclear-cut effects in the microsome plate assay.

In a quantitative respect, for benzidine, 2-acetylaminofluorene, 2-aminoanthracene and dimethylnitrosamine, obviously divergent mutagenic values were recorded with the different procedures. These differences were found to be connected with the presence of intact hepatocytes. This appeared by a comparison between mutagenicities with intact hepatocytes and with S9 prepared from disrupted hepatocytes. The results support previous recommendations that tests with intact cell metabolism should be included in a battery for screening of carcinogens in vitro.

The Salmonella/microsome assay is widely used for the detection of genotoxic agents, including carcinogens and mutagens. Because many mutagens need metabolic conversion to generate their genotoxic properties, in this test mutagenic activation in vitro is mediated by the addition of a 9000 × *g* supernatant from rat

liver (S9 fraction). It is uncertain, however, to what extent the mutagenic activation by the enzymes present in the S9 fraction approximates the biotransformation in vivo [6].

Recently we showed, for the mutagenic activation of 4-aminobiphenyl and benzidine, that metabolism by intact rat hepatocytes resembles more the biotransformation in vivo than the metabolism with a rat-liver S9 fraction [3]. It might be an interesting question in this respect whether a mutagenicity test system with intact rat hepatocytes as the metabolic factor has some value as a tool in the screening of chemicals.

In the present study, efforts were made to compare mutagenicities of some known genotoxic compounds, in the common microsome plate assay and in a test system with hepatocytes in suspension.

Materials and methods

Chemicals

Dimethylnitrosamine, benzo[*a*]pyrene, 2-acetylaminofluorene (2-AAF) and 2-aminoanthracene were purchased from Aldrich Europe (Beerse, Belgium). D-Biotin, L-histidine-HCl, collagenase type 1, nicotinamide adenine dinucleotide phosphate (NADP) disodium salt and glucose 6-phosphate (G-6-P) disodium salt were obtained from Sigma (St. Louis, MO, U.S.A.). Purified agar was from Difco Laboratories, nutrient broth No. 2 from Oxoid Ltd. (Basingstoke, U.K.). Di-*N*-butylnitrosamine and 7,12-dimethylbenzanthracene were purchased from the Eastman Kodak Company. 3-Methylcholanthrene was from Koch-Light Laboratories Ltd. 3'-Methyl-4-dimethylaminoazobenzene and 3,2'-dimethyl-4-aminobiphenyl were obtained from ICN Pharmaceuticals Inc. Benzidine and citric acid monohydrate were from Merck (Darmstadt, F.R.G.), diethylnitrosamine was from ABC (Div. of Aldrich Chemical Co. Inc., Milwaukee, U.S.A.). 2-Hydroxyethylhydrazine and cyclophosphamide were purchased from Asta (Brackwede, F.R.G.). All other chemicals used were of highest purity obtainable.

Preparation of isolated hepatocytes

The procedure was based on the methods described by Berry and Friend [2] and by Seglen [12], except for some modifications. All manipulations were performed under sterile conditions. Before the procedure, male Wistar rats were anaesthetized by an i.p. injection of 0.35 ml sodium pentobarbital (60 mg/ml distilled water). After 15 min of pre-perfusion of the liver with a Ca^{2+}-free HEPES buffer (pH 7.4), this buffer was replaced by a collagenase-containing HEPES buffer (0.06% w/v collagenase; pH 7.6) and pre-perfusion was continued for another 15 min. The perfusion rate was 40 ml/min. The crude cell suspension was treated according to the method of Seglen [12], and the final pellet was resuspended in a Ca^{2+}-containing HEPES–TES buffer solution (pH 7.6) and diluted to a density of 10×10^6 cells/ml. Trypan-blue exclusion showed the presence of about 95% viable cells.

Preparation of S9 mix from isolated hepatocytes

10 ml of a suspension of 10×10^6 liver cells/ml was centrifuged for 2 min at $1600 \times g$. The pellet was suspended in 5 ml of HEPES–TES buffer containing 2% albumin, glucose 6-phosphate (5 μmoles/ml) and NADP (4 μmoles/ml). This suspension was sonicated twice for 20 sec, with an interval of 30 sec in an MSE Ultrasonic Disintegrator (100 W) at maximal energy. During this step the suspension was cooled on ice. (After a microscopical check it turned out that all cells were broken.) Next, the sonicated suspension was centrifuged for 20 min at $9000 \times g$. To the supernatant, 5 ml of the HEPES–TES buffer containing albumin, glucose 6-phosphate and NADP was added. All manipulations were performed at 4°C and under sterile conditions.

Salmonella / hepatocyte suspension assay

The Salmonella/hepatocyte suspension assay was carried out as follows. In a shaking waterbath (210 r.p.m.), closed sterile vials (inner diameter 24 mm, height 55 mm), each containing 387.5 μl of hepatocyte suspension (10×10^6 viable cells/ml), 0.1 ml of an overnight-grown suspension of *Salmonella typhimurium* (about 1.5×10^9 bact./ml) and 12.5 μl of a solution (in DMSO) of the compound under test, were incubated at 37°C for 2 h. Next, each mixture was plated with a molten top agar containing only biotin (no histidine).

The number of revertant colonies was counted after 48 h at 37°C.

Salmonella / microsome mutagenicity plate assay

The Salmonella/microsome plate assay was performed according to Ames et al. [1] with *Salmonella typhimurium* strains hisG46, TA1535, TA1538 and TA100. We used Oxoid nutrient broth instead of Difco nutrient broth [8]. Rat-liver S9 ($9000 \times g$ supernatant) fractions were prepared from untreated male Wistar rats according to Ames et al. [1]. S9 mix contained 0.1 ml S9 per ml.

Salmonella / disrupted hepatocyte suspension assay

In this assay, *Salmonella typhimurium* and the compound under test were incubated as described for the Salmonella/hepatocyte assay. However, instead of a suspension of hepatocytes, we added 387.5 μl of S9 mix prepared from an equal amount of isolated hepatocytes used in the parallel Salmonella/hepatocyte assay. In those experiments, hepatocytes and S9 were derived from the same rat liver. Molten top agars contained only biotin (no histidine).

Results

Influence of incubation time on the mutagenicity of benzidine

In Fig. 1 the number of his^+ revertant colonies of TA1538 is shown as a function of the incubation time of benzidine with rat hepatocytes. After about 2 h of incubation, the number of revertant colonies is maximal at the lowest concentration of benzidine tested (10 μM). There is only a slow increase in the number of his^+

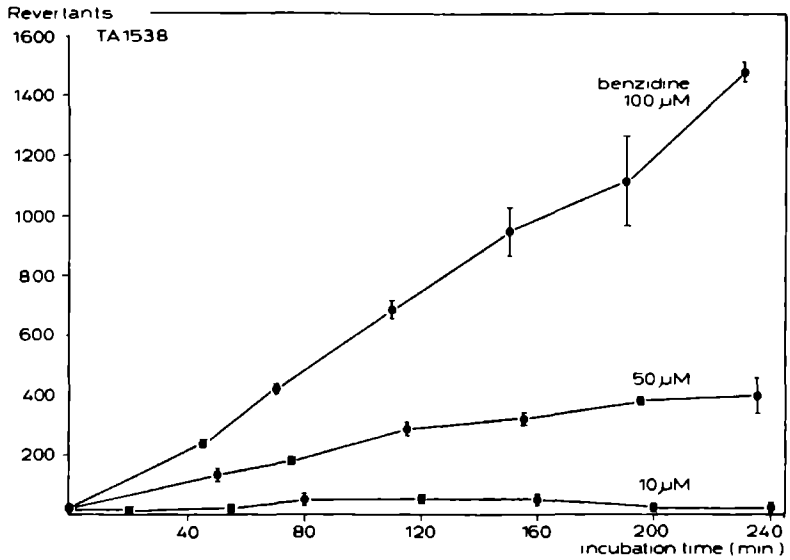

Fig. 1. Effect of incubation time on the mutagenicity of benzidine towards *Salmonella typhimurium* TA1538 in the presence of intact hepatocytes Experimental details were as mentioned in Materials and methods. Mean values ± S.E.M. of determinations in triplicate.

revertant colonies after 2 h of incubation when benzidine is tested at a concentration of 50 µM. At a benzidine concentration of 100 µM a linear correlation is found between the number of revertant colonies and the time of incubation.

These results and our findings that the viability of the rat hepatocytes ranged between 69 and 79% after 2 h of incubation and between 42 and 68% after 4 h of incubation, led us to perform the incubations during 2-h periods.

Revertants measured with the Salmonella/hepatocyte suspension and Salmonella/microsome plate assay

Aromatic amines

The mutagenicities of benzidine, 2-acetylaminofluorene and 3,2'-dimethyl-4-aminobiphenyl determined with *Salmonella typhimurium* TA1538 and TA100 are shown in Fig. 2a. No results are shown with *Salmonella typhimurium* TA1535, because none of these compounds was mutagenic towards this strain, either in the Salmonella/hepatocyte or in the Salmonella/microsome assay. Mutagenicities of 2-aminoanthracene are plotted in Fig. 2b.

These results indicate that the mutagenic properties of 2-AAF, benzidine and 3,2'-dimethyl-4-aminobiphenyl towards strains TA1538 and TA100 can be detected in the Salmonella/microsome assay as well as in the Salmonella/hepatocyte assay. The same holds true for 2-aminoanthracene, as shown in Fig. 2b.

Polycyclic aromatic hydrocarbons

Mutagenicities of benzo[*a*]pyrene, 3-methylcholanthrene and 7,12-dimethylbe-

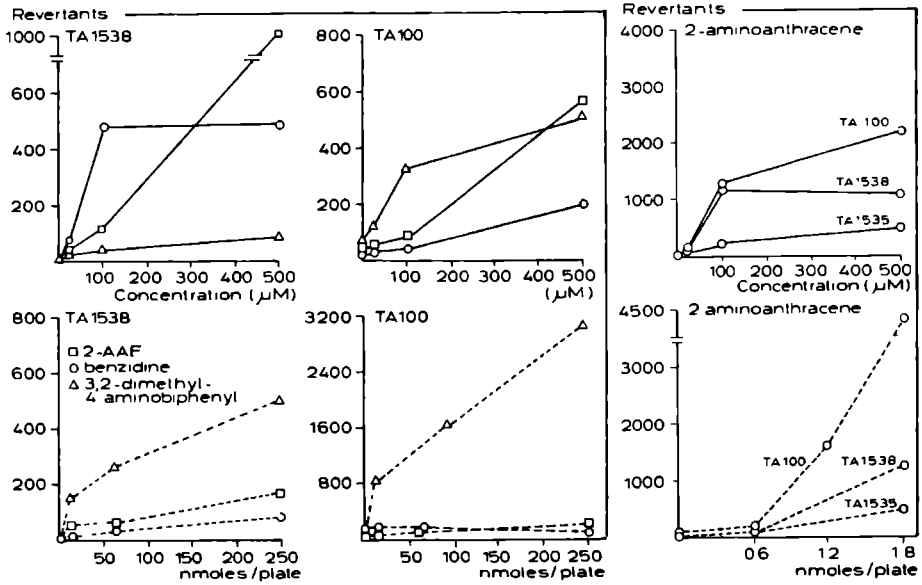

Fig. 2. (a) Mutagenicities of 2-AAF, benzidine and 3,2'-dimethyl-4-aminobiphenyl towards *Salmonella typhimurium* TA1538 and TA100 in the Salmonella/hepatocyte suspension assay (———) and in the Salmonella/microsome plate assay (- - - - - -). (b) Mutagenicities of 2-aminoanthracene towards *Salmonella typhimurium* TA1535, TA1538 and TA100 in the Salmonella/hepatocyte suspension assay (———) and in the Salmonella/microsome plate assay (- - - - - -). Experimental details were as mentioned in Materials and methods. Mean values of determinations in triplicate.

nzanthracene in the Salmonella/hepatocyte and Salmonella/microsome assays are shown in Fig. 3. The mutagenicities were determined with *Salmonella typhimurium* strains TA1535, TA1538 and TA100. Because of the limited solubility of 3-methylcholanthrene, this compound was tested up to a concentration of only 25 μM in the Salmonella/hepatocyte assay and to an amount of 125 nmoles/plate in the Salmonella/microsome assay.

The only compound that shows a clear mutagenic effect towards *Salmonella typhimurium* TA1535 in the hepatocyte assay is 7,12-dimethylbenzanthracene, whereas none of these compounds is mutagenic towards this strain in the microsome assay (Fig. 3). Benzo[*a*]pyrene is the only compound that is mutagenic in the Salmonella/hepatocyte assay towards *Salmonella typhimurium* TA1538, while 3-methylcholanthrene and 7,12-dimethylbenzanthracene are also mutagenic towards *Salmonella typhimurium* TA1538 in the Salmonella/microsome assay. Fig. 3 also shows that benzo[*a*]pyrene, 3-methylcholanthrene and 7,12-dimethylbenzanthracene are mutagenic in both assays towards *Salmonella typhimurium* TA100. 7,12-Dimethylbenzanthracene appeared to be weakly mutagenic towards *Salmonella typhimurium* TA1535 in the hepatocyte but not mutagenic in the microsome assay.

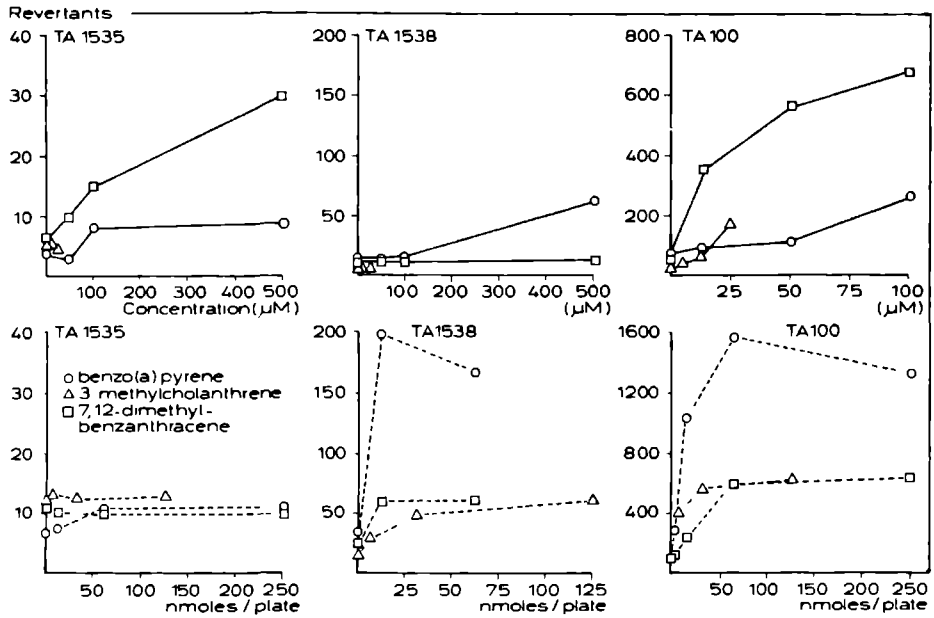

Fig. 3. Mutagenicities of benzo[*a*]pyrene, 3-methylcholanthrene and 7,12-dimethylbenzanthracene towards *Salmonella typhimurium* TA1535, TA1538 and TA100 in the Salmonella/hepatocyte suspension assay (———) and in the Salmonella/microsome plate assay (- - - - -). Experimental details were as mentioned in Materials and methods. Mean values of determinations in triplicate.

N-Nitrosoamines

The mutagenicities of dimethylnitrosamine and diethylnitrosamine in the Salmonella/hepatocyte and Salmonella/microsome assays are shown in Fig. 4. Dimethylnitrosamine is mutagenic in the hepatocyte assay towards *Salmonella typhimurium* strains hisG46, TA1535 and TA100. In the Salmonella/microsome assay it is only mutagenic to a low extent towards *Samonella typhimurium* strain hisG46. Diethylnitrosamine is mutagenic only towards *Salmonella typhimurium* hisG46 in both assays. It is mutagenic in both tests to a low extent.

We also tested the weakly carcinogenic dibutylnitrosamine in both assays. Because of its toxicity towards the bacteria it was tested up to 10 mM and 12.5 μmoles/plate in the hepatocyte and in the microsome assay, respectively. No mutagenicity of dibutylnitrosamine was detected in strains hisG46, TA1535 and TA100 in either assay.

Other compounds

Cyclophosphamide. The mutagenicity of cyclophosphamide in both the Salmonella/hepatocyte and the Salmonella/microsome assays towards *Salmonella typhimurium* TA1535 and TA100 is represented in Fig. 5. Cyclophosphamide is very mutagenic towards *Salmonella typhimurium* TA1535 and TA100 in both assays. The numbers of induced revertants are about the same. Cyclophosphamide was negative

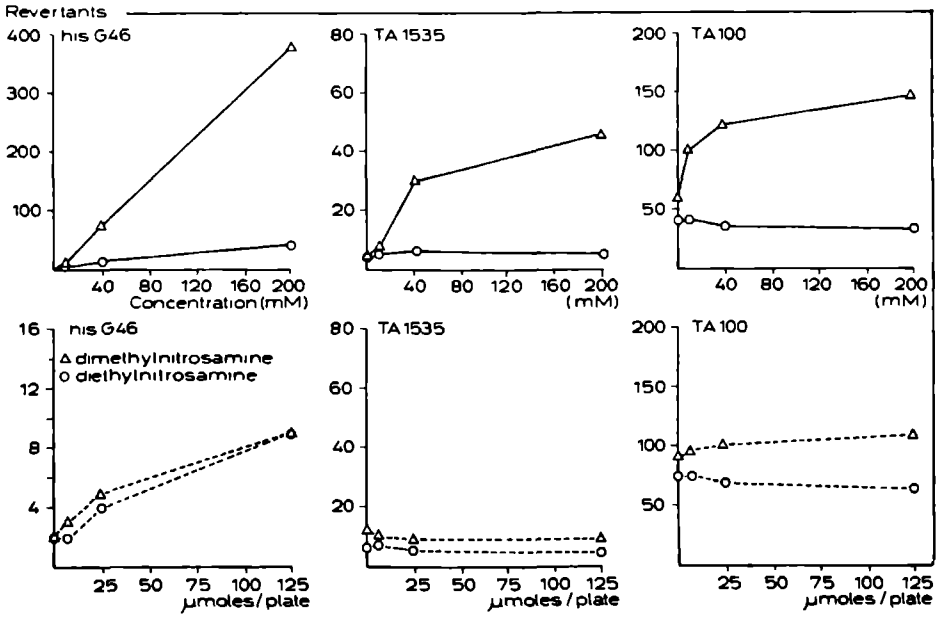

Fig. 4. Mutagenicities of dimethylnitrosamine and diethylnitrosamine towards *Salmonella typhimurium* hisG46, TA1535 and TA100 in the Salmonella/hepatocyte suspension assay (———) and in the Salmonella/microsome plate assay (- - - - - -). Experimental details were as mentioned in Materials and methods. Mean values of determinations in triplicate.

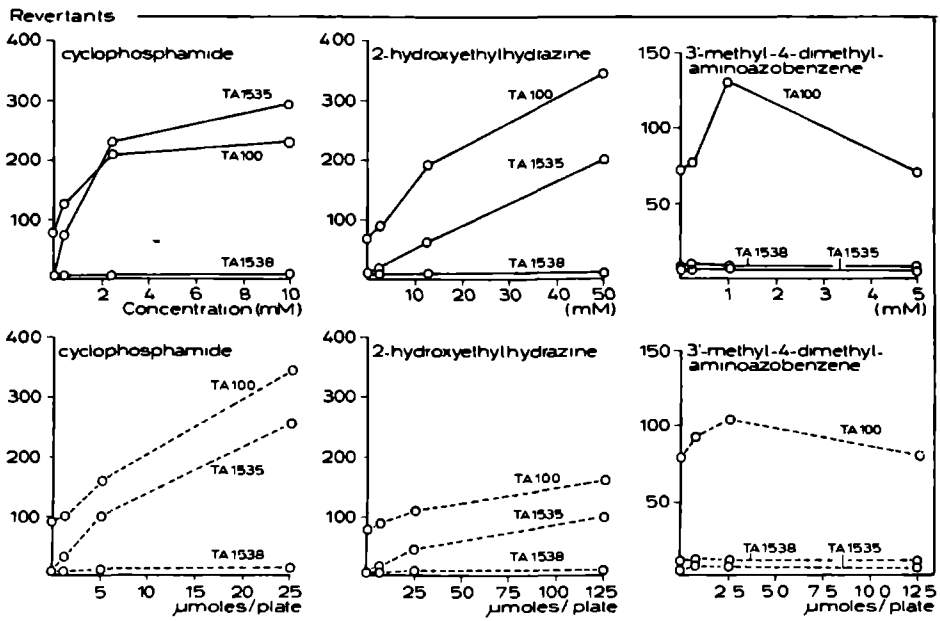

Fig. 5. Mutagenicities of cyclophosphamide, 2-hydroxyethylhydrazine and 3'-methyl-4-dimethyl-aminoazobenzene towards *Salmonella typhimurium* TA1535, TA1538 and TA100 in the Salmonella/hepatocyte suspension assay (———) and in the Salmonella/microsome plate assay (- - - - - -). Experimental details were as mentioned in Materials and methods. Mean values of determinations in triplicate.

in *Salmonella typhimurium* TA1538 in both assays.

Hydroxyethylhydrazine. Clear mutagenic responses were obtained with 2-hydroxyethylhydrazine in the two test systems with either strain TA1535 or TA100 (Fig. 5). *Salmonella typhimurium* TA1538 appeared to be insensitive for the mutagenic activity of this compound.

3'-Methyl-4-dimethylaminoazobenzene. 3'-Methyl-4-dimethylaminoazobenzene was weakly mutagenic towards *Salmonella typhimurium* TA100 in both assays. No mutagenicity of this compound was detected towards *Salmonella typhimurium* strains TA1535 andTA1538 in either assay.

Comparison of mutagenicities with intact or disrupted hepatocytes

Fig. 6 shows the mutagenicities of dimethylnitrosamine, benzidine and 2-aminoanthracene towards *Salmonella typhimurium* strains hisG46, TA1538 and TA1538, respectively. As the activating system, we used either a suspension of isolated rat hepatocytes or S9 derived from disrupted hepatocytes. All other experimental conditions in both assays were the same, except for the addition of an NADPH-generating system to the S9 preparation.

It was observed that dimethylnitrosamine and benzidine are much more mutagenic after activation by intact hepatocytes than after activation by the S9 mix towards strains hisG46 and TA1538, respectively. 2-Aminoanthracene was much more mutagenic towards TA1538 after activation with the S9 mix than after activation with intact hepatocytes.

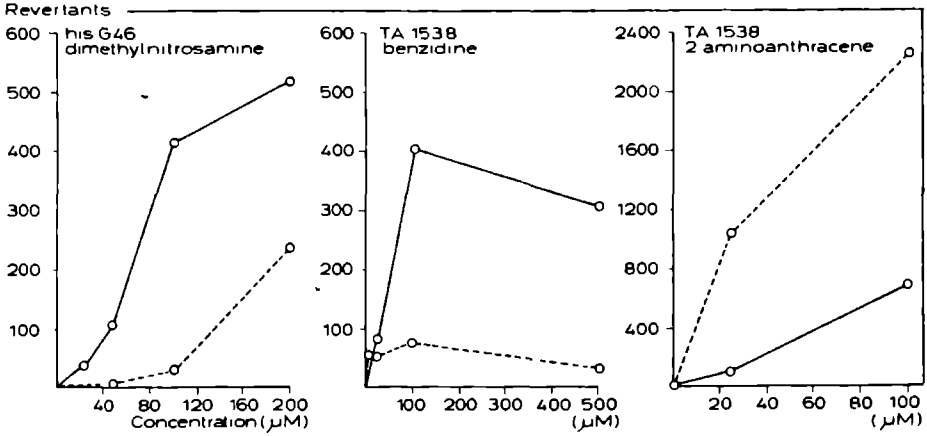

Fig. 6. Mutagenicities of dimethylnitrosamine, benzidine and 2-aminoanthracene, determined after a suspension incubation procedure, with activation by intact hepatocytes (———) or S9 from disrupted hepatocytes (- - - - -) towards *Salmonella typhimurium* hisG46, TA1538 and TA1538, respectively. Experimental details were as mentioned in Materials and methods. Mean values of determinations in triplicate.

Discussion

Previous investigations have shown that isolated hepatocytes can be considered as valuable experimental models for the study of bioactivation of premutagenic compounds, in particular those compounds that undergo complex reaction sequences [4,5]. The aim of the present study was to investigate whether a hepatocyte-mediated assay with *Salmonella typhimurium* is also useful for screening purposes.

The present results show that, in a qualitative sense, there is a conformity between the responses to most compounds tested in the Salmonella/hepatocyte and Salmonella/microsome assays. On the other hand, for some compounds qualitative differences are observed. For instance benzidine and 2-AAF are mutagenic in the Salmonella/hepatocyte but not in the Salmonella/microsome plate assay towards TA100 (Fig. 2a). Further, dimethylnitrosamine, although not mutagenic towards TA1535 in the standard plate assay, shows an obvious mutagenic effect in the hepatocyte assay towards this strain (Fig. 4). Although one is apt to believe that these differences can be explained by the intactness of intracellular relationships, it must be excluded that other differences, inherent to the assay protocol, are also responsible. In the Salmonella/hepatocyte suspension assay, mutagenic activation by rat-liver cells mainly occurs in a liquid medium. Otherwise, mutagenic activation in the Salmonella/microsome plate assay occurs in the agar medium.

To be sure that the qualitative differences observed in mutagenicity are not due to different experimental conditions but are the result of different forms of the activating system, we compared the mutagenicities of dimethylnitrosamine, benzidine and 2-aminoanthracene in the Salmonella/hepatocyte suspension assay and a Salmonella/disrupted hepatocyte suspension assay. These compounds were chosen because they showed substantial differences when tested in the Salmonella/hepatocyte suspension or Salmonella/microsome plate assay. The S9 mix used in these experiments was prepared from an equal amount of isolated hepatocytes from the same rat as used in the parallel Salmonella/hepatocyte assay. In this way we were able to study solely the effect of cellular integrity. The results indicate that the higher mutagenicity values of dimethylnitrosamine and benzidine in the Salmonella/hepatocyte assay can be ascribed to the application of intact rat hepatocytes as the metabolic factor (Fig. 6). On the other hand, the higher mutagenicity of 2-aminoanthracene as found in the Salmonella/microsome plate assay is the consequence of the use of S9 mix.

The present results of this comparative study suggest that the Salmonella/hepatocyte assay may be useful in routine testing of mutagens. This is strongly supported by the relatively high mutagenic activity of dimethylnitrosamine after metabolism by the intact hepatocytes. Although this compound is weakly mutagenic in the Salmonella/microsome assay [10], several investigators have described its substantial mutagenicity in assays in vivo [7,11]. On the basis of our previous comparison of mutagenicities of 4-aminobiphenyl and benzidine in assays in vitro with either hepatocytes or microsomal preparations, and in the intrasanguineous host-mediated assay, it is suggested that the metabolic activation by intact hepatocytes reflects the complex biotransformation in vivo [3]. Many false-positive and

false-negative results in carcinogenicity tests in vitro are believed to be explainable by inadequacies of the metabolic activation system applied in vitro [9]. Therefore, we support the recommendation that tests with intact cell metabolism, as described above, should be included in a battery for screening of carcinogens in vitro [13].

Acknowledgement

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PART 2
EXCRETION OF MUTAGENS IN URINE OF EXPERIMENTAL ANIMALS

Chapter 4

Mutagenicity of urine from rats after the administration of aromatic amines and congeners

Toxicology, 16 (1980) 113—122

THE APPEARANCE OF MUTAGENS IN URINE OF RATS AFTER THE ADMINISTRATION OF BENZIDINE AND SOME OTHER AROMATIC AMINES

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SUMMARY

The mutagenicity of urine from rats treated with benzidine or 5 other arylamines (0.25 mmol/kg; i.p.) was studied using the Ames-assay. It was found that samples of urine collected for 24 h after the administration of the carcinogens, benzidine, 4-aminobiphenyl and 2-aminonaphthalene, showed significantly mutagenic activity, whereas no mutagenicity was observed in urine after treatment with 3,3′-5,5′-tetramethylbenzidine, 2-aminobiphenyl and 1-aminonaphthalene.

Mutagenic activities were dependent on the use of either hepatic S-9 Mix or cytosol as the activating enzyme preparation. The addition of β-glucuronidase enhanced mutagenicity, except for 2-aminonaphthalene.

The appearance of mutagens in urine was studied at varying doses of benzidine and at different time-intervals after the administration. The different excretion patterns found after the activation either with S-9 Mix or with cytosolic enzyme(s) suggest the presence in urine of different types of mutagenic products.

INTRODUCTION

Durston and Ames [1] described a simple method for the detection of mutagens in urine. They demonstrated mutagenic activity in urine of rats that were treated with relatively low doses of 2-acetylaminofluorene. Independently, Commoner et al. [2] have also shown the presence of mutagenic metabolites in urine of rats fed on diets containing the carcinogens 2-acetylaminofluorene and dimethylaminoazobenzene. It was proposed that this method may have great potential for the screening of human urine to detect exposure to environmental carcinogenic agents.

In recent studies the presence of mutagens was found in urine from cigarette smokers [3,4]. Mutagens were also demonstrated in urine of patients having received therapeutic doses of the trichomonacide metronidazole or cytostatics like cyclophosphamide [5—7]. It is remarkable that nurses handling several cytostatic drugs in oncological units also exhibited mutagenicity in concentrates of their urine [8]. These mutagenic activities, however, were much lower than those found in the urine of patients treated with these drugs.

In the present study an effort has been made to further validate the urine mutagenicity test. We investigated the appearance of mutagens in the urine of rats treated with aromatic amines, some of which are carcinogenic [9,10]. It is known that a great number of metabolites of the carcinogenic arylamines appear in the urine of treated animals [11,12]. Since it is unlikely that these metabolites are excreted at the same rate, attention was paid to procedural aspects, like the time of urine sampling. In addition, the need for metabolic activation of the urinary mutagens was studied.

MATERIALS AND METHODS

Chemicals

Benzidine, 1-aminonaphthalene and D-glucose were purchased from Merck (Darmstadt, F.R.G.); 4-aminobiphenyl and 2-aminobiphenyl from Aldrich Europe (Beerse, Belgium). Bacterial β-glucuronidase, 3,3′-5,5′-tetramethylbenzidine, D-biotin and L-histidine-HCl were obtained from Sigma (St. Louis, U.S.A.); amberlite, type XAD-2 from Serva (Heidelberg, F.R.G.) and nicotinamide adenine dinucleotide phosphate (NADP) disodium salt and glucose 6-phosphate G-6-P disodium salt from Boehringer (Mannheim, F.R.G.). Purified agar and nutrient broth were purchased from Difco Laboratories (Detroit, U.S.A.). 2-Aminonaphthalene was purchased from Fluka (Buchs, Switzerland), saccharo-1,4-lactone from Calbiochem (Los Angeles, U.S.A.) and citric acid monohydrate from J.T. Baker Chemicals (Deventer, The Netherlands). All other chemicals used were of highest purity obtainable.

Animals

Male Wistar rats weighing about 200 g were purchased from TNO (Rijswijk, The Netherlands). The animals were housed individually in stainless steel metabolism cages, designed for the separate collection of urine and feces. The rats had free access to water and food (Hope Farms, Woerden, The Netherlands). Aromatic amines, dissolved or suspended in olive oil (under nitrogen to prevent oxidation) were injected intraperitoneally.

Urine samples were collected for 24 h, unless stated otherwise, and stored at −20°C until assayed. Before they were assayed, the individual samples were completed to 15 ml and sterilized by filtration through 0.2 μm membrane filters.

Mutagenicity testing

Mutagenicity of the urine samples was determined using the *Salmonella typhimurium* strains TA 1538 and TA 100 [13]. 0.1 ml of a nearly full-grown suspension of the bacteria (2×10^9 bact./ml) was added to the topagar, containing 0.3 ml of diluted urine, and was supplied with varying volumes of an activating enzyme system. As indicated elsewhere, the activating enzyme system consisted of one or more of the following components:

(a) 0.5 ml S-9 Mix, prepared according to Ames et al. [13], containing 50 μl hepatic 9000 *g* supernatant from phenobarbital induced rats and a NADPH-generating system.

(b) 0.4 ml liver cytosol, equivalent to 100 mg fresh liver, derived from non-induced rats.

(c) 0.1 ml of a sterile β-glucuronidase solution (6500 U/ml).

In one experiment urine samples were collected every 5 h during a 50 h period, they were stored at $-20°C$ until assayed. Each 5-h sample was put through a column with a 4-cm^3 bed volume of amberlite XAD-2, washed with 1.5 ml of water to remove histidine [3] and the adsorbed material was eluted with 10 ml of aceton. The eluate was evaporated to dryness under nitrogen at 60°C and the residue was dissolved in 0.85 ml of dimethylsulphoxide. 0.1 ml of this solution was added per plate and assayed for mutagenicity with the *Salmonella typhimurium* tester strain TA 1538 in the presence of an activating enzyme system.

RESULTS

Effect of various activating systems.

Several combinations of activating enzymes were studied to detect mutagenic activity in urine of rats treated with benzidine. The results are summarized in Table I. It can be seen that in the absence of an activating system no substantial mutagenicity was found. The addition of the conventionally used microsomal oxidizing, S-9 Mix, resulted in the appearance of a great number of revertant colonies. Further, addition of β-glucuronidase in combination with S-9 Mix gave an increase of about 40%. This may indicate the presence in urine of glucuronidated benzidine metabolites, which after hydrolysis, can be activated through microsomal oxidation to the proximate mutagens. Addition of saccharo-1,4-lactone, a specific inhibitor of the β-glucuronidase activity [14], completely counteracted the enhancement of mutagenicity in the presence of β-glucuronidase but did not significantly influence the activation by the S-9 Mix alone.

On the other hand, as is shown in Table I, addition of a hepatic cytosol fraction resulted in the appearance of a considerable number of revertant colonies, which increased about 10-fold by the action of β-glucuronidase. Addition of saccharo-1,4-lactone had no influence on the activating capacity of the cytosol fraction. Further, it is shown that the action of β-glucuronidase alone was insufficient to form the proximate mutagens.

When the NADPH-generating system (which delivers the essential cofactor

TABLE I

EFFECT OF DIFFERENT ACTIVATING SYSTEMS ON MUTAGENICITY OF URINE FROM BENZIDINE-TREATED RATS[a] DETECTED WITH *S. TYPHIMURIUM* TA 1538

Additions[c]	Revertants/plate[b]			
	None	β-glucuronidase	Saccharo-1,4-lactone	β-glucuronidase + saccharo-1,4-lactone
S-9 Mix	4430 ± 340	6200 ± 510	4760 ± 140	3730 ± 515
S-9 Mix without NADP/G-6-P	112 ± 4	524 ± 22		
Cytosol	595 ± 33	6500 ± 220	744 ± 48	
None	59 ± 3	76 ± 2		
Spontaneous	42 ± 5			

[a]Rats received 250 μmol/kg i.p., 24-h urine was diluted to 15 ml. Samples of 0.3 ml were plated in the presence of 0.1 ml TA 1538 suspension (2× 10⁹ bact./ml).
[b]Mean values (±S.E.M.) of 3 measurements of the same urine.
[c]Additions to the topagar (per plate): S-9 Mix, 0.5 ml; Cytosol, 0.4 ml of 105 000 *g* supernatant from 25% rat liver homogenate; β-glucuronidase, 650 units dissolved in 0.1 ml water; saccharo-1,4-lactone, final conc. 3 mM.

for the monooxygenase activity) was left out of the S-9 mixture, the activating capacity disappeared almost totally. It is therefore excluded that the activation which is brought about by liver cytosol, represents an artifact due to contamination by microsomal particles.

Influence of the dose of benzidine

Rats were injected intraperitoneally with increasing amounts of benzidine. Twenty-four-hour urine samples were collected and tested for mutagenicity with S-9 Mix or liver cytosol, either with or without the addition of β-glucuronidase. The number of revertant colonies/plate was plotted as a function of the dose of benzidine (Fig. 1a,b). It can be seen in Fig. 1a that the relative increase in mutagenicity caused by the action of β-glucuronidase is highest at low doses of benzidine. This may indicate that at the lower doses a greater fraction is excreted as glucuronides that, after being hydrolysed, need an oxidative activation to become proximate mutagens. Possibly, at increasing benzidine concentrations the glucuronyl transferases or the mechanisms involved in the transport of the glucuronides become saturated. At higher doses (above 0.1 mmol/kg) the graphs of Fig. 1a show a reasonably linear relationship between urinary mutagenicity and dosage. In contrast, the urinary mutagenicity that results from a combined activation with β-glucuronidase and cytosol enzymes, is independent of the dose at high doses of benzidine (Fig. 1b).

Time course of the appearance of mutagens in urine

The excretion of mutagenic products in urine of rats after the adminis-

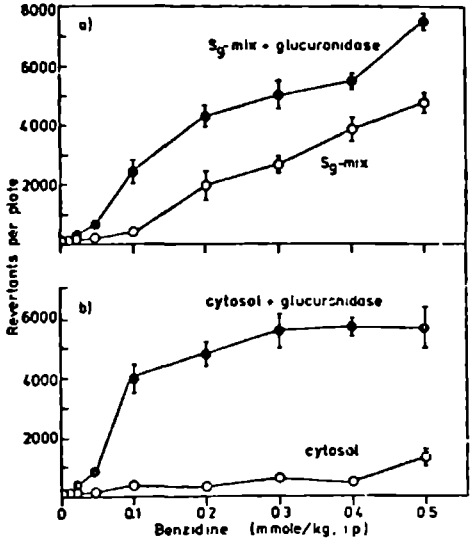

Fig. 1. (a,b) Influence of the dose of benzidine on the urinary mutagenicity, as detected with different activating enzyme preparations. Experimental details were as mentioned in Materials and Methods.

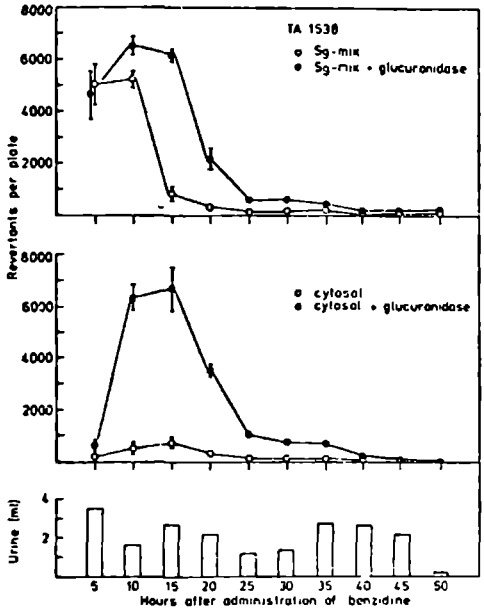

Fig. 2. (a,b,c) Time course of the appearance of mutagens in urine, as detected with different activating enzyme preparations. Experimental details were as mentioned in Materials and Methods.

TABLE II (a)

MUTAGENIC ACTIVITY IN URINE OF RATS TREATED WITH SEVERAL ARYLAMINES

Treatment[b]	Number of *his*$^+$ revertants/plate[a]					
	Urine and tester strain TA 1538 added to the topagar with:[c]					
	—	β-glucuronidase	S-9 Mix	S-9 Mix + β-glucuronidase	Cytosol	Cytosol + β-glucuronidase
Benzidine	66 ± 6	86 ± 7	4200 ± 120	6200 ± 100	480 ± 31	7200 ± 320
3,3'-5,5'-Tetramethylbenzidine	46 ± 8	47 ± 9	47 ± 4	46 ± 3	35 ± 4	32 ± 1
4-Aminobiphenyl	47 ± 2	49 ± 3	100 ± 10	189 ± 14	55 ± 6	277 ± 8
2-Aminobiphenyl	42 ± 2	51 ± 10	51 ± 5	58 ± 7	48 ± 5	49 ± 5
2-Aminonaphthalene	46 ± 3	45 ± 8	85 ± 6	80 ± 7	42 ± 6	36 ± 3
1-Aminonaphthalene	44 ± 2	47 ± 2	61 ± 4	50 ± 6	43 ± 2	45 ± 4

[a]Mean values (± S.E.M.) of 3 measurements of the same urine.

[b]Rats received 250 μmol/kg i.p., 24-h urine was diluted to 15 ml. Samples of 0.3 ml were plated in the presence of 0.1 ml TA 1538 suspension (2×10^9 bact./ml).

[c]Additions to the topagar (per plate): S-9 Mix, 0.5 ml; cytosol, 0.4 ml of 105 000 *g* supernatant from 25% rat liver homogenate; β-glucuronidase, 650 units dissolved in 0.1 ml water.

TABLE II (b)

MUTAGENIC ACTIVITY IN URINE OF RATS TREATED WITH SEVERAL ARYLAMINES

Treatment[b]	Number of *his*$^+$ revertants/plate[a]					
	Urine and tester strain TA 100 added to the topagar with:[c]					
	—	β-glucuronidase	S-9 Mix	S-9 Mix + β-glucuronidase	Cytosol	Cytosol + β-glucuronidase
Benzidine	212 ± 12	281 ± 10	7040 ± 200	7300 ± 630	424 ± 27	7400 ± 320
3,3′-5,5′-Tetramethylbenzidine	188 ± 2	240 ± 11	210 ± 14	208 ± 7	204 ± 5	241 ± 16
4-Aminobiphenyl	199 ± 14	260 ± 23	444 ± 13	1210 ± 45	201 ± 5	822 ± 27
2-Aminobiphenyl	220 ± 11	239 ± 10	248 ± 16	293 ± 17	192 ± 7	216 ± 9
2-Aminonaphthalene	181 ± 14	230 ± 4	782 ± 14	850 ± 47	199 ± 17	219 ± 31
1-Aminonaphthalene	222 ± 4	234 ± 4	230 ± 16	234 ± 14	213 ± 16	252 ± 9

[a]Mean values (± S.E.M.) of 3 measurements of the same urine.

[b]Rats received 250 μmol/kg i.p., 24-h urine was diluted to 15 ml. Samples of 0.3 ml were plated in the presence of 0.1 ml TA 100 suspension (2×10^9 bact./ml).

[c]Additions to the topagar (per plate): S-9 Mix, 0.5 ml; cytosol, 0.4 ml of 105 000 *g* supernatant from 25% rat liver homogenate; β-glucuronidase, 650 units dissolved in 0.1 ml water.

tration of benzidine was studied as a function of the time. Thus benzidine, 0.25 mmol/kg, was injected i.p. and urine samples were collected at intervals of 5 h. The urine samples were concentrated on a XAD-2 column and tested for mutagenicity as mentioned above.

Comparison of the graphs in Figs. 2a and b reveals that the mutagenic products, which can be activated with S-9 Mix, appeared earlier in the urine than those which exert their mutagenicity after activation with liver cytosol or combined β-glucuronidase and liver cytosol.

The volumes of the urine portions consecutively produced during the 5-h periods are depicted in Fig. 2c. It is apparent that the excretion patterns of the urinary mutagens are independent of the quantities of urine produced in each period.

Comparison of six aromatic amines

The mutagenic activity in urine of rats that were treated with equal doses of different aromatic amines (0.25 mmol/kg; i.p.) was assayed in 24 h urine samples, using the bacterial strains TA 1538 and TA 100. Three pairs of structurally related arylamines were studied. Both S-9 Mix and hepatic cytosol, either with or without the addition of β-glucuronidase, were used for the activation of mutagenic compounds. The results are given in Table II. No mutagenic activity was detected with TA 100 and TA 1538 in the urine of rats treated with the compounds: 3,3'-5,5'-tetramethylbenzidine, 2-aminobiphenyl and 1-aminonaphthalene. However, urine of rats treated with the carcinogenic structure analogues, benzidine, 4-aminobiphenyl and 2-aminonaphthalene, showed significant mutagenic values. The urine of animals given 4-aminobiphenyl or 2-aminonaphthalene was much less mutagenic than the urine from the benzidine-treated rats. Further, it was found that, in contrast with benzidine and 4-aminobiphenyl, administration of 2-aminonaphthalene did not lead to urinary excretion of product(s) that can be converted to active mutagen(s) by the action of cytosolic enzymes.

DISCUSSION

These results clearly demonstrate that the administration to rats of benzidine or some other carcinogenic amines results in the excretion of certain products in the urine that are not directly mutagenic to *Salmonella typhimurium*, but become mutagenic after the addition of activating enzymes. Although the excretion products following the administration of benzidine to rats have not been identified in the present study, there are good reasons to assume that these are for the most part metabolic products of benzidine and not benzidine itself. For instance, the total mutagenic activity of rat urine collected for 2 days after the injection of a certain dose of benzidine, greatly surpasses the mutagenic potential of the same amount of benzidine when tested with the same bacterial strains in vitro, using metabolic activation with S-9 Mix.

It was further shown that different enzymes can be used for the metabolic

activation of the urinary products, namely microsomal monooxygenase or cytoplasmic enzyme(s), either with or without the concomitant application of β-glucuronidase.

The increase in mutagenicity by the presence of β-glucuronidase indicates that part of the mutagenic metabolites appear in the urine as glucuronides. This is in accordance with the findings of Durston and Ames [1] and Commoner et al. [2] who detected mutagens in the urine of rats fed 2-AAF. Durston and Ames [1] detected most mutagenicity when urine was incubated with S-9 Mix in the presence of β-glucuronidase. Commoner et al. [2] detected most mutagenicity with S-9 Mix in an ether extract of urine preincubated with β-glucuronidase and Taka-diastase.

We now present evidence that not only the action of the oxidative enzymes present in the S-9 Mix can lead to the formation of mutagenic products, but the cytosolic liver fraction also appears to contain enzyme(s) capable of converting certain urinary products of benzidine into proximate mutagens.

The data depicted in Figs. 1a and b suggest that the dose-dependency of the urinary mutagenicity resulting from an activation with β-glucuronidase and S-9 Mix differs from that found after treatment of the urine samples with β-glucuronidase and liver cytosol.

From Fig. 2 it may be concluded that the metabolites that were activated with S-9 Mix appeared in urine earlier after application of benzidine than those activated with cytosol. Almost all detectable mutagens in urine were excreted within 24 h after application. Kellner et al. [15] found that after intravenous administration of 0.2 mg [^{14}C]benzidine/kg body wt to male Wistar rats 17% of the radioactivity appeared in urine. The urinary excretion was almost completed within 1 day. This is in good agreement with our results.

From the results shown in Figs. 1 and 2 it may be concluded that treatment of rats with benzidine results in the appearance in urine of different classes of compounds, some of which require oxidative, and others non-oxidative metabolism to produce mutagenic activity.

One of the enzymes present in rat liver cytosol that could be responsible for the activation of urinary metabolites is *N,O*-acyltransferase. *N*-Hydroxydiacetylbenzidine that has weak mutagenic properties by itself, was found to be activated by the action of *N,O*-acyltransferase [16]. This gives rise to the presumption that the mutagens we detected with the aid of cytosol are *N*-hydroxyacetylderivatives of benzidine and 4-aminobiphenyl, which are mainly excreted in urine as glucuronides. *N*-Hydroxyacetylderivatives of benzidine were found in the urine of people fed benzidine [17]. The urinary metabolite of 4-aminobiphenyl detected as mutagen after activation with cytosol in the presence of β-glucuronidase might be the glucuronide of *N*-hydroxy-4-acetylaminobiphenyl, which was identified by Miller et al. [11] to be a metabolite in rat urine after treatment with 4-aminobiphenyl.

From a comparison of mutagenic activities in urine of rats treated with different aromatic amines (Table II), it appears that only those compounds which have obvious carcinogenic potencies give rise to the excretion of muta-

gens in urine. The mutagenic activity of urine from rats given benzidine was much higher than that in the urine from 4-aminobiphenyl treated rats. This is remarkable because 4-aminobiphenyl appeared to be more mutagenic when tested in vitro with TA 1538 and TA 100 using rat liver S-9 Mix as the activating system. Further studies are in progress now to explain this discrepancy.

ACKNOWLEDGEMENTS

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INVOLVEMENT OF NON-OXIDATIVE ENZYMES IN MUTAGENIC ACTIVATION OF URINE FROM RATS, GIVEN BENZIDINE AND SOME OTHER AROMATIC AMINES

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SUMMARY

Urinary metabolites of rats treated with benzidine and some other genotoxic aromatic amines became mutagenic in the Ames assay after activation with liver cytosol from rat, mouse and guinea pig. Most of the mutagenic metabolites appeared in urine as glucuronides. Strong evidence was found that *N,O*-acyltransferase is responsible for the mutagenic activation by rat liver cytosol. The inhibitory effect of paraoxon and sodium fluoride indicates that the activation by mouse liver cytosol is due to the action of deacetylase. Mutagenic activation by guinea pig liver cytosol seemed to be mediated in part by deacetylase. The metabolite activated by these enzymes most likely is a glucuronidated, N-acetylated, N-hydroxylated product.

INTRODUCTION

In our previous work it was shown that after treatment of adult male Wistar rats with some aromatic amines, mutagens could be detected in their urine, using various activating systems in the Ames assay [1]. It was remarkable that the use of rat liver cytosol as an activating system in testing urine of benzidine treated rats, caused a considerable number of revertant colonies. In the presence of β-glucuronidase the mutagenic activation was enhanced, whereas the action of β-glucuronidase alone was insufficient to

Abbreviations: G-6-P, glucose 6-phosphate; NADPH, nicotinamide adenine dinucleotide phosphate; TLC, thin-layer chromatography.

form the proximate mutagens. This activation was suggested to be non-oxidative.

Two possibilities of non-oxidative enzymatic conversion of metabolites of aromatic amines which enhance mutagenicity for *Salmonella typhimurium* have been described. First there is an enzymatic deacetylation reaction which can be inhibited by paraoxon and sodium fluoride [2]. For example *N*-hydroxy-2-acetamidofluorene has been shown to be many times less mutagenic than its deacetylated product *N*-hydroxy-2-aminofluorene [3,4]. The second enzymatic reaction that gives rise to an increase in mutagenicity of metabolites of aromatic amines is a reaction mediated by *N,O*-acyltransferase. This enzyme is able to transfer an *N*-acetyl group from an *N*-acetyl-*N*-arylhydroxylamine to the oxygen to form the *O*-acetyl derivative of the *N*-arylhydroxylamine. Due to the action of this enzyme in the presence of *N*-hydroxy-2-acetamidofluorene or *N*-hydroxy-diacetylbenzidine an increased mutagenicity to *Salmonella typhimurium* was detected [5,6]. In the investigation presented in this paper we studied the nature of the mutagenic activation of rat urinary benzidine metabolites by liver cytosol from rats, mice and guinea pigs. In addition we present data about some other aromatic amines which can be detected as mutagens in urine from rats after activation by liver cytosol.

MATERIALS AND METHODS

Chemicals

Benzidine, sodium fluoride and ammoniumsulfate were purchased from Merck (Darmstadt, F.R.G.). 2-Acetamidofluorene, 2-aminofluorene, 2-aminoanthracene, 4-aminobiphenyl and diethyl-*p*-nitrophenylphosphate (paraoxon) were from Aldrich Europe (Beerse, Belgium). 2,7-Diaminonaphthalene was obtained from ICN Pharmaceuticals, Inc., Sephadex G-100 from Pharmacia (Uppsala, Sweden). Bacterial β-glucuronidase, D-biotin and L-histidine-HCl, were obtained from Sigma (St. Louis, U.S.A.). Amberlite, type XAD-2 was purchased from Serva (Heidelberg, F.R.G.). Nicotinamide adenine dinucleotide phosphate (NADP) disodium salt and glucose 6-phosphate (G-6-P) disodium salt were purchased from Boehringer (Mannheim, F.R.G.). Purified agar was from Difco Laboratories, nutrient broth No. 2 from Oxoid Ltd. (Basingstoke, England) and citric acid monohydrate from J.T. Baker Chemicals (Deventer, The Netherlands). All other chemicals used were of highest purity obtainable.

Animals

Male Wistar rats weighing about 200 g were purchased from TNO (Rijswijk, The Netherlands). The animals were housed individually in stainless steel metabolism cages, designed for the separate collection of urine and feces. The rats had free access to water and food (Hope Farms, Woerden, The Netherlands). Aromatic amines, dissolved or suspended in olive oil (under nitrogen to prevent oxidation) were injected intraperitoneally (250 μmol/kg).

Urine samples were collected for 24 h and stored at −20°C until assayed. Before testing, the individual samples were completed to 15 ml and sterilized by filtration through 0.2-μm membrane filters.

Mutagenicity testing

Mutagenicity of the urine samples was determined using the *Salmonella typhimurium* strains TA1538 and TA100 [7]. 0.1 ml of an overnight grown suspension of the bacteria (±1.5 × 10^9 bact./ml) was added to the topagar, containing 0.1 ml of diluted urine, and was supplied with an enzyme system. Unless otherwise indicated, this enzyme system consisted of 0.4 ml liver cytosol, equivalent to 100 mg fresh liver, derived from non-induced male Wistar rats, male Swiss mice or male albino guinea pigs, and/or 0.1 ml of a sterile β-glucuronidase solution (1500 U/ml).

Effect of paraoxon and sodium fluoride

In the study of the effects of paraoxon and sodium fluoride on the mutagenic activation of urine of benzidine treated rats by liver cytosol from rats, mice and guinea pigs, we used the method described by Okuno et al. [8] with some modifications: 0.4 ml liver cytosol was 15 min preincubated at 37°C with various paraoxon/sodium fluoride concentrations and then mixed with 0.1 ml of urine of a benzidine treated rat, 0.1 ml of a bacteria suspension (TA1538) and 0.1 ml of a β-glucuronidase solution (1500 Units/ml). This mixture was further incubated at 37°C for 20 min. After addition of 2 ml of topagar the mixture was plated.

Identification of the activating enzyme in rat liver cytosol

Partial purification of the activating enzyme was applied to 4 g of wet rat liver material following the method described by King [9] with some slight modifications. We used a column of Sephadex G-100 (3 × 100 cm) and a flowrate of 3.7 ml/h. 0.7 ml fractions were collected. The UV-absorbance of the eluate was monitored continuously at 254 nm.

Activating capacity was determined in the Ames assay by adding 0.1 ml of the filter sterilized fractions to 0.1 ml of urine of benzidine treated rats, 0.1 ml of a bacterial suspension (TA1538) and 0.1 ml of a β-glucuronidase solution (1500 U/ml).

Mutagenicity testing of benzidine metabolites separated by thin-layer chromatography (TLC)

A 24-h urine sample of a benzidine treated rat was applied to a column with a 4 cm^3 bed volume of Amberlite XAD-2. Next the adsorbed material was eluted with 10 ml of acetone. The eluate was evaporated to dryness under nitrogen at 60°C and the residue was dissolved in 0.6 ml of ethanol. Eighteen samples of 12 μl of this solution were applied to TLC-plates. Glass plates (5 × 20 cm) coated with a 0.25-mm thick layer of silicagel 60 F_{254} obtained from Merck (Darmstadt, F.R.G.) were employed. Butanol/acetic acid/water (4 : 1 : 1; v/v) was used as solvent system. Metabolites on TLC-plates were visualized under UV-light of 254 nm. Separated metabolites were

scraped off. Eighteen corresponding metabolite fractions were combined and dissolved in 2.4 ml of dimethylsulphoxide. Silicagel rests were removed by centrifugation. 0.1 ml of this solution was added per plate and assayed for mutagenicity with the *Salmonella typhimurium* tester strain TA1538 in the presence of an activating system.

RESULTS

Mutagenic activation of urine of benzidine treated rats with liver cytosol from rats, mice or guinea pigs

The effect of liver cytosol from rats, mice or guinea pigs on the mutagenic activity in urine of rats treated with benzidine was studied. The results are shown in Table I. It can be seen that in the absence of cytosol no substantial mutagenicity was found. Addition of hepatic cytosol from rats or guinea pigs resulted in an enhancement in the number of revertant colonies. A number that increased many times by the addition of β-glucuronidase. Mutagenicity was hardly detectable in the presence of mouse liver cytosol, whereas after the addition of β-glucuronidase an obvious mutagenic activity was observed. β-glucuronidase alone did not result in a remarkable number of revertants.

Effect of paraoxon and sodium fluoride on the deacetylating enzymes in liver cytosol from rat, mouse or guinea pig

Figure 1 shows the effect of paraoxon and sodium fluoride on the mutagenic activation of urinary benzidine metabolites by liver cytosol from rats, mice or guinea pigs. It is shown that the activation by rat liver cytosol was not counteracted by paraoxon or sodium fluoride, whereas the activation by

TABLE I

EFFECT OF LIVER CYTOSOL FROM RAT, MOUSE AND GUINEA PIG ON MUTAGENICITY OF URINE FROM BENZIDINE TREATED RATS[a] DETECTED WITH *SALMONELLA TYPHIMURIUM* TA1538

Additions[c]	Revertants/plate[b]	
	None	β Glucuronidase
None	7 ± 1	18 ± 1
Rat liver cytosol	80 ± 7	854 ± 9
Mouse liver cytosol	16 ± 1	139 ± 5
Guinea pig liver cytosol	39 ± 6	701 ± 16
Background	7 ± 1	

[a] Rats received 250 μmol/kg i.p., 24 h urine was diluted to 15 ml. Samples of 0.1 ml were plated in the presence of 0.1 ml TA1538 suspension (1.5 × 10^9 bact./ml).

[b] Mean values (±S.E.M.) of 3 measurements of the same urine.

[c] Additions to the topagar (per plate): cytosol, 0.4 ml of 105 000 *g* supernatant from 25% liver homogenate; β-glucuronidase, 150 units dissolved in 0.1 ml water.

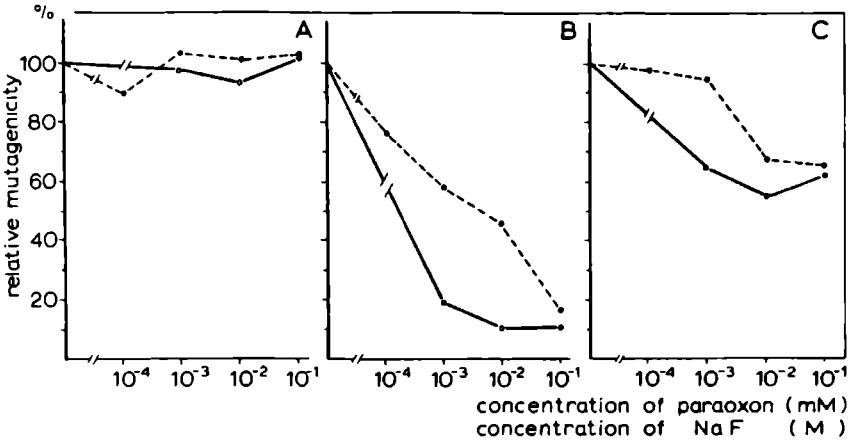


Fig. 1. Influence of paraoxon and sodium fluoride (broken line) on the cytosol mediated mutagenicity of urine of benzidine treated rats. Liver cytosol from rats (A), mice (B) and guinea pigs (C) was used in the presence of β-glucuronidase. Experimental details were as mentioned in Materials and Methods.

mouse liver cytosol almost completely disappeared in the presence of paraoxon or sodium fluoride. The inhibition of the activation by guinea pig liver cytosol seems to have an intermediate character.

The activating rat liver cytosolic enzyme

A deacetylation reaction that can be inhibited by paraoxon and sodium fluoride did not seem to play a role in mutagenic activation of urinary benzidine metabolites by rat liver cytosol. The question arises whether an activation by *N,O*-acyltransferase is involved. In order to clarify this question we subjected the rat liver cytosol to gel filtration chromatography by using the same method as King applied for purification of *N,O*-acyltransferase [9]. We did not measure *N,O*-acyltransferase activity directly, but tested the fractions only on their mutagenic activating capabilities. It was interesting to see that the fractions having the highest acyltransferase activity as measured by King corresponded with those fractions in our study having the highest mutagenic activating capability. It is tempting therefore to conclude that the mutagenic activation is due to the action of *N,O*-acyltransferase (Fig. 2).

Cytosolic activation of urinary benzidine metabolites separated by thin-layer chromatography (TLC)

Results of the cytosolic activation of benzidine metabolites, separated by TLC, into mutagens are shown in Table II. Although the activation by rat liver cytosol and mouse liver cytosol is different, as can be seen from Fig. 1, the metabolite they activate is the same.

It was noted that no mutagenic effects were detected with liver cytosol without further addition of β-glucuronidase. Presumably, the (pre)mutagenic metabolite excreted into the urine is a glucuronide.

TABLE II

MUTAGENIC ACTIVATION OF BENZIDINE METABOLITES SEPARATED BY THIN-LAYER CHROMATOGRAPHY

R_F value	Number of *his*$^+$ revertants/plate[a]			
	TLC-fractions[b] and tester strain TA1538 added to the topagar with:[c]			
	Rat liver cytosol	Rat liver cytosol + β-glucuronidase	mouse liver cytosol + β-glucuronidase	Guinea pig liver cytosol + β-glucuronidase
0 (origin)	3 ± 1	3 ± 1	4 ± 1	7 ± 1
0.32	4 ± 1	25 ± 8	4 ± 1	19 ± 1
0.37	6 ± 3	310 ± 28	188 ± 20	256 ± 38
0.41	14 ± 3	23 ± 2	13 ± 2	20 ± 2
0.49	21 ± 4	23 ± 5	18 ± 4	22 ± 2
0.54	20 ± 3	20 ± 5	15 ± 1	31 ± 4
0.63	11 ± 4	12 ± 4	11 ± 2	20 ± 2
Background	5 ± 1			

[a] Mean values (±S.E.M.) of 3 measurements of the same urine.
[b] Details about preparation of different TLC-fractions are mentioned in Materials and Methods.
[c] Additions to the topagar (per plate): cytosol, 0.4 ml of 105 000 *g* supernatant from 25% liver homogenate; β-glucuronidase, 150 units dissolved in 0.1 ml water.

Comparison of benzidine with some other aromatic amines

Five other aromatic amines besides benzidine, given to rats at a dose of 0.25 mmol/kg, were found to result in the appearance of mutagens detectable after addition of liver cytosol. Mutagenic activities of urine of treated rats after addition of liver cytosol from rats, mice or guinea pigs are given in Table IIIa and IIIb for the tester strain TA1538 and TA100, respectively. In accordance with the findings shown in Fig. 1 these results suggest that cytosol liver fractions from rats, mice and guinea pigs contain different activating enzyme systems. Mutagenic urinary metabolites from rats given benzidine are well detectable after activation with liver cytosol from rats or guinea pigs in the presence of β-glucuronidase, whereas they also might be detected with liver cytosol from mice in the presence of β-glucuronidase. Mutagenic activity in the urine of 4-aminobiphenyl treated rats was poorly detectable whatever liver cytosol was used for the activation. Treatment of rats with 2-aminofluorene or 2-acetamidofluorene resulted in the appearance of urinary mutagens which were detectable after activation with liver cytosol in the presence of β-glucuronidase. In this respect liver cytosol from guinea pig was more active than that from rat or mouse. In the case of 2-aminoanthracene detection of mutagenic activity is possible after activation with liver cytosol from guinea pigs in the presence of

TABLE III (a)

MUTAGENIC ACTIVATION OF URINE OF RATS TREATED WITH ARYLAMINES BY LIVER CYTOSOL FROM RAT, MOUSE OR GUINEA PIG

Treatment[b]	Number of *his*$^+$ revertants/plate[a]							
	Urine and tester strain TA1538 added to the topagar with:[c]							
	—	β-Glucuronidase	Rat liver cytosol	Rat liver cytosol + β-glucuronidase	Mouse liver cytosol	Mouse liver cytosol + β-glucuronidase	Guinea pig liver cytosol	Guinea pig liver cytosol + β-glucuronidase
4-Aminobiphenyl	8 ± 1	7 ± 1	11 ± 4	25 ± 1	11 ± 2	13 ± 1	8 ± 1	26 ± 2
2-Aminofluorene	37 ± 4	36 ± 7	29 ± 2	548 ± 19	27 ± 2	431 ± 18	142 ± 3	1000 ± 40
2-Acetamidofluorene	37 ± 4	35 ± 5	35 ± 4	493 ± 23	35 ± 3	360 ± 21	128 ± 7	718 ± 28
2-Aminoanthracene	8 ± 2	9 ± 2	19 ± 3	90 ± 10	12 ± 2	28 ± 3	67 ± 1	150 ± 11
2,7-Diaminonaphthalene	7 ± 1	7 ± 1	39 ± 2	13 ± 1	61 ± 8	15 ± 4	893 ± 27	689 ± 13

[a] Mean values (±S.E.M.) of 3 measurements of the same urine.

[b] Rats received 250 μmol/kg i.p., 24-h urine was diluted to 15 ml. Samples of 0.1 ml were plated in the presence of 0.1 ml TA1538 suspension (1.5×10^9 bact./ml).

[c] Additions to the topagar (per plate): cytosol, 0.4 ml of 105 000 *g* supernatant from 25% liver homogenate; β-glucuronidase 150 units dissolved in 0.1 ml water.

TABLE III (b)

MUTAGENIC ACTIVATION OF URINE OF RATS TREATED WITH ARYLAMINES BY LIVER CYTOSOL FROM RAT, MOUSE OR GUINEA PIG

Treatment[b]	Number of *his*$^+$ revertants/plate[a]							
	Urine and tester strain TA100 added to the topagar with:[c]							
	—	β-Glucuronidase	Rat liver cytosol	Rat liver cytosol + β-glucuronidase	Mouse liver cytosol	Mouse liver cytosol + β-glucuronidase	Guinea pig liver cytosol	Guinea pig liver cytosol + β-glucuronidase
Benzidine	162 ± 7	145 ± 14	165 ± 16	915 ± 90	146 ± 7	366 ± 9	204 ± 13	1030 ± 20
4-Aminobiphenyl	153 ± 2	155 ± 14	145 ± 5	141 ± 7	127 ± 12	178 ± 9	197 ± 8	298 ± 23
2-Aminofluorene	145 ± 6	152 ± 6	99 ± 20	436 ± 35	117 ± 8	725 ± 22	303 ± 26	1370 ± 40
2-Acetamidofluorene	139 ± 14	136 ± 11	91 ± 8	453 ± 27	107 ± 2	481 ± 21	135 ± 12	1100 ± 50
2-Aminoanthracene	145 ± 13	189 ± 14	209 ± 12	781 ± 30	137 ± 6	263 ± 34	796 ± 41	1470 ± 160
2,7-Diaminonaphthalene	155 ± 6	138 ± 7	105 ± 9	123 ± 1	114 ± 2	114 ± 11	303 ± 18	249 ± 7

[a] Mean values (±S.E.M.) of 3 measurements of the same urine.

[b] Rats received 250 μmol/kg i.p., 24-h urine was diluted to 15 ml. Samples of 0.1 ml were plated in the presence of 0.1 ml TA100 suspension (1.5×10^9 bact./ml).

[c] Additions to the topagar (per plate): cytosol, 0.4 ml of 105 000 *g* supernatant from 25% liver homogenate; β-glucuronidase 150 units dissolved in 0.1 ml water.

β-glucuronidase, to a lesser extent after activation with rat liver cytosol in the presence of β-glucuronidase and only poorly after activation with liver cytosol from mice in the presence of β-glucuronidase. In contrast, mutagens in urine of rats treated with 2,7-diaminonaphthalene were found in the absence of β-glucuronidase; guinea pig liver cytosol is the most suitable activating system in this case.

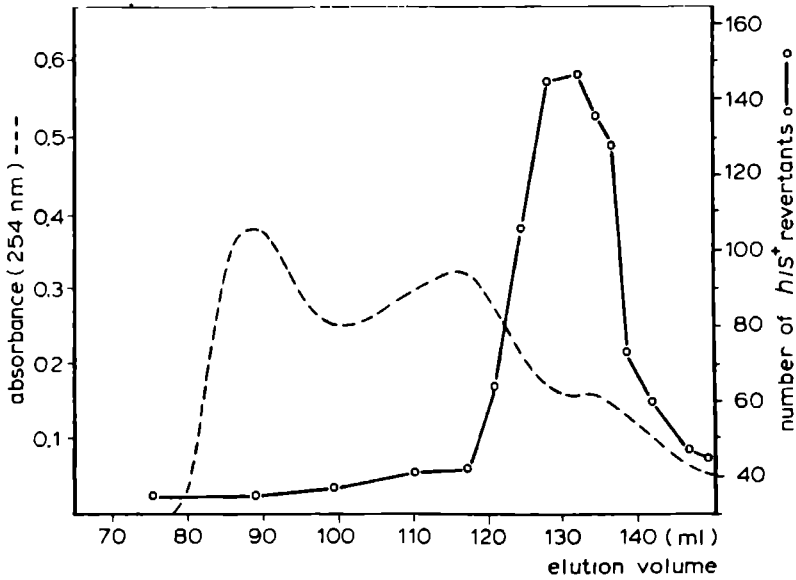
The use of *Salmonella typhimurium* strain TA100 as detection organism for the activated mutagens led to similar results.

DISCUSSION

Urinary mutagenic metabolites of some genotoxic aromatic amines were detected by application of the Ames assay using liver cytosol from rat, mouse or guinea pig as the activating system. The considerable increase in mutagenicity by the presence of β-glucuronidase might indicate that a major part of the mutagenic metabolites appear in urine as glucuronides except for the metabolites of 2,7-diaminonaphthalene.

It is interesting that treatment with the bifunctional aromatic amines, benzidine and 2,7-diaminonaphthalene leads to the production of urine with higher mutagenic potency than when the rats were treated with 4-aminobiphenyl or 2-aminonaphthalene. Treatment of rats with a dose up to 2 mmol/kg of 2-aminonaphthalene did not result in the appearance of mutagens in urine that are detectable after activation by liver cytosol in the presence or absence of β-glucuronidase.

With respect to the mutagenic activation by the enzymes present in liver cytosol, an obvious species difference in the sensitivity for the deacetylase inhibitors, paraoxon and sodium fluoride, was observed (Fig. 1). The activating capacity in mouse liver cytosol can be inhibited almost completely by paraoxon (10^{-4} M) or sodium fluoride (10^{-1} M). It is known that paraoxon does not affect the *N,O*-acyltransferase catalyzed mutations in Salmonella [5,10]. Nor does sodium fluoride inhibit the enzymatic transacetylation [11]. This may suggest that in the case of mouse liver cytosol a deacetylation rather than a transacetylation is responsible for the activation of the mutagens. On the other hand, mutagenic activation of the urinary benzidine metabolites by liver cytosol from the rat can not be inhibited by paraoxon or sodium fluoride (Fig. 1). King [9] conclusively demonstrated the presence of *N,O*-acyltransferase in rat liver cytosol and emphasized the role of this enzyme in the generation of genotoxic products from *N*-hydroxy-2-acetamidofluorene. The data of Fig. 2 shows that the enzyme in rat liver cytosol responsible for the mutagenic activation of urinary benzidine metabolite(s), probably is the same as characterized by King [9] being *N,O*-acyltransferase. Activation of the urinary benzidine metabolites by guinea pig liver cytosol seemed to be, at least in part, the result of the action of deacetylase (Fig. 1). These results also correspond with data from other investigators. Schut and Thorgeirsson [12] did not find *N*-hydroxy-2-acetamidofluorene deacetylase activity in rat



Fi . 2. Gel filtration chromatography of the activating enzyme present in rat liver cytosol. A partially purified fraction of rat liver cytosol was chromatographed on Sephadex G-100. The mutagenic activating capacity of the fractions is expressed as the number of revertants. Experimental details were as mentioned in Materials and Methods.

liver cytosol. Irving [2,13] detected some deacetylating capacity in liver cytosol from guinea pig. Bartsch et al. [14] reported a substantial transacetylating activity in rat liver cytosol whereas little or no activity was observed in liver cytosol from guinea pig or mouse [11,15,16].

From the foregoing it may be concluded that the mutagenic activity of the urine of benzidine treated rats can be achieved by different enzymes. However, as is suggested by the data of Table II, only 1 premutagenic urinary metabolite was found. Therefore, one may speculate about the nature of this metabolite. The mutagenic product from this metabolite formed through the action of either *N,O*-acyltransferase (rat liver cytosol) or deacetylase (mouse liver cytosol) presumably contains a hydroxylated amino function. Such metabolites are known mutagenic substances for *Salmonella typhimurium* [17]. Studies are in progress now to identify the premutagenic urinary benzidine metabolite. Most likely this is a N-acetylated, N-hydroxylated and glucuronidated derivative. Trol et al. [18] and Belman et al. [19] found *N*-hydroxyacetyl derivatives of benzidine in the urine of people given benzidine, thereby showing the ability of humans to produce this kind of urinary metabolites.

The Ames assay on urine samples is suggested to be useful in the detection of human exposure to genotoxic substances [20,21]. We propose that when exposure to arylamines is involved, urinary mutagenicity tests should be carried out using an activating system containing cytosol and β-glucuronidase.

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METABOLISM OF BENZIDINE-BASED DYES AND THE APPEARANCE OF MUTAGENIC METABOLITES IN URINE OF RATS AFTER ORAL OR INTRAPERITONEAL ADMINISTRATION

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SUMMARY

The azo reduction and acetylation *in vitro* and the mutagenic activation *in vivo* of three azo dyes were studied. In the presence of rat-liver 9000 *g* supernatant benzidine was released from direct black 38 and direct brown 95, whereas hardly any benzidine was produced during incubation of direct blue 6. Incubation of benzidine with isolated rat hepatocytes resulted in the appearance of diacetylbenzidine. No diacetylbenzidine was formed during incubation of benzidine with rat-liver 9000 *g* supernatant, unless the cofactor for the acetylation reaction, acetyl coenzyme A, was added to the incubation medium. Isolated rat hepatocytes were capable to produce diacetylbenzidine from direct black 38, direct blue 6 or direct brown 95 without supplementation with acetyl coenzyme A.

Administration of benzidine, direct black 38 or direct brown 95 to rats resulted in the appearance of mutagenicity in urine. For direct black 38 significantly higher mutagenicity values were found in urine after oral administration than after intraperitoneal treatment. Such differences were not observed for benzidine and direct brown 95.

The results demonstrate that rat liver has a considerable capacity to reduce azo compounds. Nevertheless, for some compounds, like direct black 38, extrahepatic enzymes, most likely present in the intestinal flora, may also play a substantial role in the azo cleavage.

Submitted to Toxicology

INTRODUCTION

Benzidine-based dyes are widely used in dyeing of textiles such as cotton, silk, wool and nylon (1). They are also used in plastics, paper and leather, in aquous printing inks and in wood stains. Some case-control mortality studies indicate an increased risk of developing bladder-cancer after occupational exposure to benzidine-based dyes via inhalation, ingestion or skin absorption (2,3). The carcinogenic potential of these dyes has been attributed on one hand to the presence of residual benzidine in the dyes (4), on the other hand to benzidine that has been formed metabolically as a result of reduction *in vivo* of the azo bonds. Studies in animals given these benzidine-based dyes showed the appearance of benzidine and benzidine metabolites in urine (1,5,6). Tanaka et al. (7) demonstrated that oral administration of the benzidine-based dyes congo red or direct black 38 to rats resulted in mutagenicity of the urine extracts, when tested in the Salmonella/microsome assay. This mutagenicity was ascribed to the presence of benzidine metabolites in urine.

In a review concerning the metabolism of azo compounds, Walker (8) suggested that it may be concluded that the intestinal azo-reducing systems were considerably more active and non-specific than the hepatic azo reductase in animals. For a benzidine-based dye like direct black 38 it was suggested by Cerniglia et al. (9) that anaerobic intestinal bacteria may play a significant role in the metabolism. The data presented by Martin and Kennelly (10) suggest that mammalian liver may play only a minor or negligible role in the azo reduction of dyes derived from benzidine. This conclusion was drawn from experiments *in vitro*. Liver preparations, like microsomes, generally differ in activity of enzymes involved in the biotransformation and concentrations of cofactors from the intact liver *in vivo*.

In the present investigation we studied the metabolism of some benzidine-based dyes (Fig. 1) and measured the mutagenicity of rat urine after either intraperitoneal or oral administration of these compounds. Since several studies suggest that the overall metabolism in the hepatocyte is fairly representative for the biotransformation *in vivo* (11,12,13), we have investigated azo reduction by intact hepatocytes.

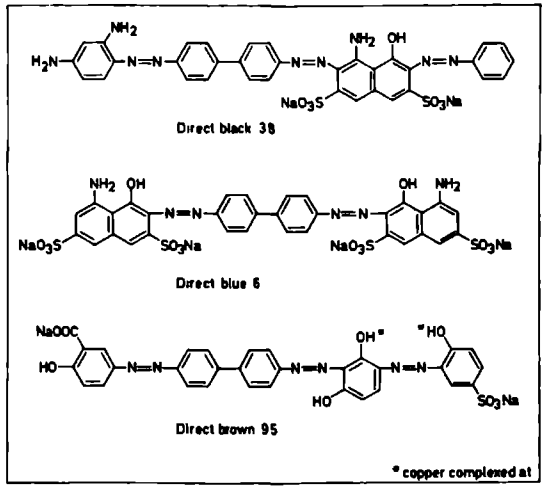

Fig. 1 Molecular structures of direct black 38, direct blue 6 and direct brown 95.

MATERIALS AND METHODS

Chemicals

Direct black 38, direct blue 6 and direct brown 95 were obtained from Pechiney Ugine Kuhlmann (Amsterdam, The Netherlands). It was ascertained that no residual benzidine was present in the samples of the dyes. Benzidine (BD) was purchased from Merck (Darmstadt, F.R.G.). D-Biotin, L-histidine-HCl, collagenase type I, nicotinamide adenine dinucleotide phosphate (NADP) disodium salt and glucose-6-phosphate (G-6-P) disodium salt were purchased from Sigma (St. Louis, U.S.A.). Acetyl coenzyme A was obtained from Boehringer (Mannheim, F.R.G.). Purified agar was from Difco Laboratories, nutrient broth No. 2 from Oxoid Ltd. (Basingstoke, England), and amberlite type XAD-2 from Serva (Heidelberg, F.R.G.). Monoacetylbenzidine (MAB) was purchased from ICN Pharmaceuticals, Inc. N,N'-diacetylbenzidine (DAB) was synthetized according to Johnson et al. (14). All other chemicals used were of highest purity obtainable.

Animals

SPF Wistar rats weighing about 200 g had free access to water and RMH food (Hope Farms, Woerden, The Netherlands).

Collection of urine samples

The SPF rats were housed individually in stainless steel metabolism cages, designed for the separate collection of urine and feces.

Solutions of benzidine, direct black 38 and direct brown 95 in tragacanth (2%) (under nitrogen to prevent oxidation) were injected intraperitoneally or given per os (250 µmol/kg).

Urine samples were collected for 24 h and stored at -20°C until assayed. Before they were assayed the individual samples were completed to 15 ml and sterilized by filtration through 0.2 µm membrane filters.

Preparation of isolated hepatocytes

The procedure was based on the methods described by Berry and Friend (15) and by Seglen (16), except for some modifications. Before the procedure, male Wistar rats were anaesthetized by an i.p. injection of 0.35 ml sodium pentobarbital (60 mg/ml distilled water). After 15 min of pre-perfusion of the liver with a Ca^{2+}-free HEPES buffer, this buffer was replaced by a collagenase-containing HEPES buffer (0.06% w/v collagenase) and pre-perfusion was continued for another 15 min. The perfusion rate was 25 ml/min. The crude cell suspension was treated according to the method of Seglen (16), and the final pellet was resuspended in a Ca^{2+}-containing HEPES-TES buffer solution and diluted to a density of 5 x 10^6 cells/ml. Trypan-blue exclusion showed the presence of about 95% viable cells.

Incubation of the dyes and isolation of the metabolites

200 µl of a solution of the testcompound, was incubated for 4 hrs at 37°C (210 r.p.m.) in the presence of either 10 ml of rat-liver 9000 *g* supernatant, prepared as S9 mix according to Maron and Ames (17) or 10^8 hepatocytes in 10 ml of HEPES-TES buffer solution. The incubation was stopped on ice. Next, the cells were sonicated twice for 15 seconds and the suspension was centrifuged for 10 min at 4000 *g*. The supernatant was put through a column with a 4 cm^3 bed volume of amerlite XAD-2, washed

with plenty of water and the adsorbed material was eluted with 10 ml of acetone. The eluate was evaporated to dryness under nitrogen at 60°C and the residue was dissolved in 100 µl of solvent B.

High-performance liquid chromatography (HPLC)

Aliquots of the solubilized residue were injected into the 0.02 ml sample loop of a Pye Unicam HPLC equipped with a 3 XP pump, a variable wavelength detector and a 10 mV Kipp recorder. The stainless steel column was 150 mm long i.d. 4.6 mm and filled with Lichrosorb RP 18. The flow rate was 1 ml/min and the wavelength was adjusted to 280 nm. Simultaneously with sample injection, the following solvent programme was commenced:
10 min linear gradient from 100% solvent A (80% aquapur, 10% 5M HAc/NaAc (pH = 5.0), 10% methanol) to 80% solvent A/20% solvent B (90% methanol, 10% 1M HAc/NaAc (pH = 5.0)). 25 min linear gradient from 80% A/20% B to 55% A/45% B. 5 min linear gradient from 55% A/45% B to 100% B. Finally 5 min solvent B and return to A.

Mutagenicity testing

Mutagenicity of the urine samples was determined using the *Salmonella typhimurium* strain TA1538 (17). 0.1 ml of an overnight grown suspension of the bacteria ($\pm$ 1.5 x 10^9 bact./ml) was added to the topagar, containing 0.3 ml of diluted urine, and was supplied with an enzyme system. The activating enzyme system consisted of either 0.5 ml S9 mix, prepared according to Maron and Ames (17), containing 50 µl hepatic 9000 *g* supernatant from phenobarbital induced rats and a NADPH-generating system, in the presence of 0.1 ml of a sterile β-glucuronidase solution (1500 U/ml), or 0.4 ml liver cytosol, equivalent to 100 mg fresh liver, derived from non-induced rats, in the presence of 0.1 ml of a sterile β-glucuronidase solution 1500 U/ml (18,19).

RESULTS

Azo reduction of direct black 38, direct blue 6 and direct brown 95 by rat-liver 9000 g supernatant

The incubation of direct black 38, direct blue 6 and direct brown 95 with rat-liver 9000 *g* supernatant resulted in the appearance of benzidine. Neither

monoacetylbenzidine nor diacetylbenzidine was detected. Fig. 2 shows the amounts of benzidine that were released after incubation of the dyes at various concentrations at 37°C for 4 hrs. Incubations of direct black 38 and direct brown 95 at concentrations over approximately 5 mM resulted in a decreased product formation. Hardly any benzidine was produced during incubation of direct blue 6.

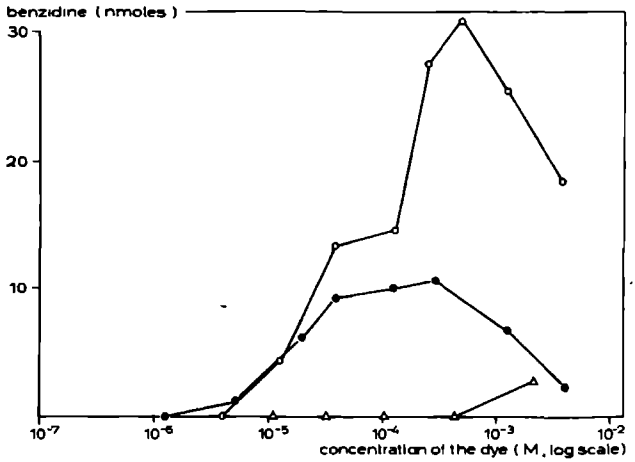

Fig. 2 The amount of benzidine measured after a 4 h incubation at 37°C of 10.2 ml of a solution of direct black 38 (●——●), direct blue 6 (Δ——Δ), or direct brown 95 (o——o) with rat-liver 9000 g supernatant. Experimental details were as mentioned in Materials and Methods.

Pretreatment of rats with phenobarbital (75 mg/kg per day for 4 days) resulted in an increase of hepatic cytochrome P450 of about 200 per cent. No enhancement of the formation of benzidine was observed when 9000 *g* liver supernatant from induced animals was incubated with direct brown 95. Further fractionation of the hepatic 9000 *g* supernatant by centrifugation at 105000 *g* revealed that the reducing enzyme activity was present in the 105000 *g* supernatant.

In vitro acetylation of benzidine by isolated rat hepatocytes and 9000 g supernatant

Incubations of low concentrations of benzidine with isolated rat hepatocytes resulted in the appearance of considerable amounts of diacetylbenzidine (table 1). The amount of monoacetylbenzidine formed after

Table 1

METABOLISM OF BENZIDINE INTO MONOACETYLBENZIDINE AND DIACETYLBENZIDINE BY ISOLATED RAT HEPATOCYTES[a]

	Concentration (μM)[b]			
	Before incubation	After incubation		
Hepatocytes/ml	BD	BD	MAB	DAB
0	540	540	n.d.	n.d.
10^7	540	440 ± 75	150 ± 23	22 ± 4
10^7	110	n.d.	4 ± 0.6	100 ± 17
10^7	27	n.d.	n.d.	25 ± 4

[a]Incubation was at 37°C for 2 hours. Further experimental conditions were as described in Materials and Methods.

[b]Mean values (± S.E.M.); n.d. not detectable.

incubation of benzidine with rat-liver 9000 *g* supernatant was negligible, whereas diacetylbenzidine was not detected. When, however, the incubation medium was supplemented with the cofactor for the acetylation reaction, acetyl coenzyme A, enzymes of the rat-liver 9000 *g* supernatant were capable to convert benzidine into monoacetylbenzidine and diacetylbenzidine (table 2).

Table 2

METABOLISM OF BENZIDINE INTO MONOACETYLBENZIDINE AND DIACETYLBENZIDINE BY RAT-LIVER 9000 *g* SUPERNATANT IN THE PRESENCE OF ACETYL COENZYME A[a]

Concentration (μM)[b]				
Before incubation		After incubation		
BD	Acetyl coenzyme A	BD	MAB	DAB
5.4	0	5 ± 0.9	0.08 ± 0.02	n.d.
5.4	37	n.d.	0.7 ± 0.12	3.6 ± 0.6
5.4	124	n.d.	0.11 ± 0.02	5.0 ± 0.9
5.4	370	n.d.	n.d.	5.0 ± 0.9

[a]Incubation was at 37°C for 4 hours. 10.2 ml incubation mixture contained 1 ml 9000 *g* supernatant equivalent to 250 mg rat liver. Experimental details were as mentioned in Materials and Methods.

[b]Mean values (± S.E.M.); n.d. not detectable.

Metabolism of direct black 38, direct blue 6 and direct brown 95 by isolated rat hepatocytes

After incubation of direct black 38, direct blue 6 or direct brown 95 with isolated rat hepatocytes, we found diacetylbenzidine. Neither benzidine nor monoacetylbenzidine was detected. Apparently, by the azo-bond reduction in these dyes, benzidine was released and immediately acetylated by the hepatocytes.

Fig. 3 shows the production of diacetylbenzidine that was formed during incubation of direct black 38, direct blue 6 and direct brown 95 with rat hepatocytes at 37°C for 4 hrs. The substrate concentrations at which diacetylbenzidine formation is optimal, are rather different for the 3 dyes. This suggests different affinities of the dyes for the metabolizing systems involved. At increasing concentrations of the dyes the metabolism was inhibited.

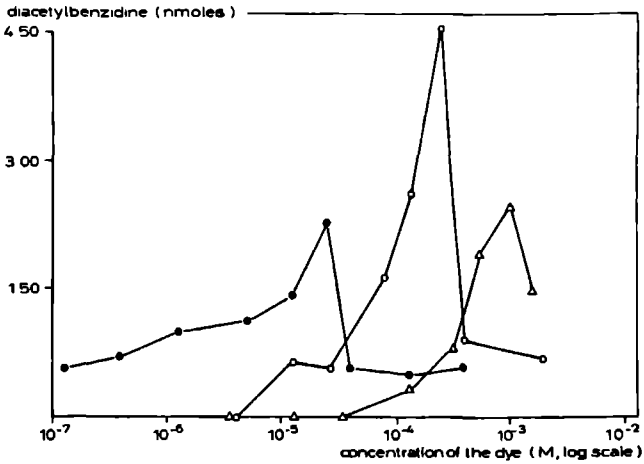

Fig. 3 The amount of diacetylbenzidine measured after a 4 h incubation at 37°C of 10.2 ml of a solution of direct black 38 (●——●), direct blue 6 (Δ——Δ), or direct brown 95 (o——o) with isolated rat hepatocytes. Experimental details were as mentioned in Materials and Methods.

Excretion of mutagenic metabolites in urine of rats

In animal studies it was investigated whether administration *in vivo* of the benzidine-based dyes leads to formation and excretion of acetylated benzidine metabolites, which are known to be mutagenic towards *Salmonella typhimurium* TA1538 after appropriate activation. Table 3 shows mutagenic activities in urine from rats having received benzidine, direct black 38 or direct brown 95, either intraperitoneally or per os. Mutagenic activation of the urinary metabolites was carried out by two different systems; S9 mix in the presence of β-glucuronidase, or rat liver cytosol in the presence of β-glucuronidase (18,19). Highest mutagenicity values were found after treatment of the rats with benzidine. For this compound and for direct brown 95 no remarkable differences in the appearance of urinary mutagens after administration via different routes were observed. On the other hand, for direct black 38 relatively high mutagenicity values were found in urine of rats after oral administration. This difference is most pronounced after

Table 3

MUTAGENICITY OF URINE FROM RATS AFTER ADMINISTRATION OF BENZIDINE DIRECT BLACK 38 OR DIRECT BROWN 95, EITHER INTRAPERITONEALLY OR PER OS

Treatment[c]	Number of his$^+$ revertants/plate[a]	
	Urine and tester strain TA1538 added to the topagar with[b]:	
	S9 mix + β-glucuronidase	rat liver cytosol + β-glucuronidase
benzidine i.p.		
experiment 1	6980 ± 50	9390 ± 590
experiment 2	8730 ± 280	6150 ± 600
experiment 3	9230 ± 300	11000 ± 330
benzidine p.o.		
experiment 1	6010 ± 380	7290 ± 530
experiment 2	9110 ± 120	9460 ± 280
experiment 3	8740 ± 350	10300 ± 1070
direct black 38 i.p.		
experiment 1	346 ± 20	748 ± 30
experiment 2	327 ± 4	113 ± 3
experiment 3	243 ± 4	141 ± 5
direct black 38 p.o.		
experiment 1	930 ± 61	5670 ± 510
experiment 2	647 ± 15	4790 ± 190
experiment 3	763 ± 8	3770 ± 460
direct brown 95 i.p.		
experiment 1	450 ± 33	1230 ± 20
experiment 2	215 ± 27	1020 ± 60
experiment 3	126 ± 20	637 ± 25
direct brown 95 p.o.		
experiment 1	133 ± 23	527 ± 36
experiment 2	210 ± 23	1050 ± 70
experiment 3	131 ± 12	714 ± 3
untreated	23 ± 3	30 ± 5

[a] Mean values (± S.E.M.) of 3 measurements of the same urine.

[b] Additions to the topagar (per plate): S9 mix, 0.5 ml; rat liver cytosol, 0.4 ml of 105000 *g* supernatant from 25% rat liver homogenate; β-glucuronidase, 150 Units dissolved in 0.1 ml water.

metabolic activation with rat liver cytosol.

DISCUSSION

The detection of benzidine after incubation of direct black 38 or direct brown 95 with hepatic 9000 *g* supernatant, indicates that enzymes capable of azo dye reduction are present in this liver preparation (Fig. 2). After incubation of direct blue 6 hardly any benzidine was detected. Further findings suggested that cytochrome P-450 is not rate-limiting in the reduction of these dyes into benzidine. In addition, it was observed that the enzymes involved in the reduction of direct brown 95 were present in the 105000 *g* supernatant fraction of rat liver. Therefore, most likely the enzyme responsible for the reduction of direct brown 95 is the cytosolic azoreductase described by Huang et al. (20). This is not in agreement with the findings of Martin and Kennelly (10), who found rat liver microsomes, from phenobarbital-pretreated animals, capable to reduce the azo groups of a number of dyes, among which the benzidine-based dyes direct black 38 and congo red.

In table 1 we showed that intact hepatocytes - in contrast with 9000 *g* supernatant - were able to convert benzidine into acetylated derivatives. The inability of the 9000 *g* supernatant fraction to acetylate benzidine, can be explained by the absence of acetyl coenzyme A (table 2).

Lazear et al. (21) and Tanaka et al. (7) showed that monoacetylbenzidine and diacetylbenzidine are much more mutagenic than the parent compound benzidine. In a previous study (22) we demonstrated that benzidine was much more mutagenic after activation with isolated rat hepatocytes than after activation with rat liver 9000 *g* supernatant. On the basis of the present results, this is most likely attributable to the capability of rat hepatocytes to acetylate benzidine.

In the animal experiments (table 3) it is shown that after administration of benzidine (250 µmoles/kg) much more mutagenicity appeared in rat urine than after the administration of equal doses of direct black 38 or direct brown 95. Tanaka et al. (7) suggested that urinary mutagenicity after administration of such azo dyes is caused by urinary metabolites of benzidine that is released by azo reduction.

Another mutagenic product that is formed during reduction of direct black

38, is 1,2,4-triaminobenzene. Administration to rats of 1,2,4-triaminobenzene resulted in the appearance of mutagenicity in urine. However, after metabolic activation with S9 mix in the presence of β-glucuronidase higher values were observed than after activation with rat liver cytosol in the presence of β-glucuronidase (data not shown). The number of revertants obtained with 1,2,4-triaminobenzene amounted only about one per cent of that after an equal dose of benzidine. This finding supports the concept that the urinary mutagenicity is caused by the presence of metabolites of benzidine.

In regard of genotoxic activation of azo dye compounds, much emphasis has been led on the role of intestinal metabolism (2,9). It is generally thought that after oral exposure these substances are rapidly reduced with the aid of azo-cleaving enzymes of the bacterial flora. The benzidine released after azo reduction will be absorbed and subsequently be activated by enzymes of the liver. If the liver only has a poor reducing capacity, this concept includes that genotoxic metabolites are formed in a less degree if the azo compounds enter the organism parenterally, thereby avoiding the metabolism of the gut.

From a comparison of mutagenicities found in urine after either intraperitoneal or oral administration of direct black 38, it is obvious that indeed most mutagens appeared in urine after oral administration of this dye (table 3). After intraperitoneal administration, urinary mutagenicity may be considered as the result of azo reduction, mainly occurring in the liver. Intestinal metabolism seems to result in an extra contribution to the urinary mutagenicity due to this dye. This is in accordance with the results of Cerniglia et al. (9), who showed that anaerobic incubations *in vitro* of direct black 38 with rat intestinal microorganisms leads to an effective reduction of the azo bonds. On the other hand, for direct brown 95 we did not find a significant difference between the urinary mutagenicities after either intraperitoneal or oral administration. It can be noticed that direct brown 95 was reduced more rapidly by liver 9000 *g* supernatant than direct black 38.

Further studies are necessary to answer the question which factors determine whether the first step in the toxification process of azo compounds takes place in the gut or in the liver. Undoubtedly, the rate of intestinal absorbtion which depends on physico-chemical properties (such as lipophilicity) plays an important role. The results described in this paper

demonstrate that the liver has a substantial capacity to reduce azo compounds.

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PART 3
URINARY MUTAGENICITY AFTER EXPOSURE OF HUMANS TO MUTAGENIC SUBSTANCES

Chapter 5

Mutagenicity and thioether concentration of urine due to smoking

Int. Arch. Occup. Environ. Health 43, 159—166 (1979)

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Original Works

Thioether Concentration and Mutagenicity of Urine from Cigarette Smokers

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Summary. Urinary thioether compounds, such as mercapturic acids, can be considered as nontoxic end products of potentially alkylating agents. On the other hand, urinary mutagenicity may represent the excretion of potential mutagens that have not definitively been detoxified by the organism. It is suggested that a combined urinary thioether and mutagenicity test may be useful in monitoring people occupationally exposed to potentially alkylating compounds, in particular to mixtures of these chemicals. Exposure to cigarette smoke, containing several known mutagens and carcinogens, is expected to interfere with the test results.

The excretion of mutagens and thioethers was determined in urine of smokers and nonsmokers. Smokers excrete more mutagens and thioether compounds than nonsmokers. Further, it was found that the urinary mutagenicity and thioether level are significantly related to the amount of cigarettes smoked.

Key words: Urinary thioethers – Mercapturic acids – Mutagenicity of urine – Cigarette smoking – Potentially alkylating agents – Exposure test

Many studies have emphasized the role of endogenous sulfhydryl-containing compounds, particularly glutathione (GSH), in the detoxication of chemicals such as mutagens and carcinogens that act through the covalent binding of reactive metabolites (Jollow et al., 1974; Jaeger et al., 1974; Mitchell et al., 1976; van Doorn et al., 1978). The conjugates that are formed by the reaction of the electrophilic metabolites with the nucleophilic GSH, are subsequently excreted through the bile or in the urine, commonly as mercapturic acids (N-acetylcysteine derivatives) (Chasseaud, 1973, 1976).

Previously, we suggested that the appearance of mercapturic acids or other thioether compounds in urine might be a useful indication of exposure to potentially alkylating agents (Seutter-Berlage et al., 1977). Accordingly, it was

found that employees of chemical plants, exposed to complex mixtures of chemicals, excreted more thioether compounds than their colleagues working in administrative departments. Recently, Vainio et al. (1978) also reported raised concentrations of thioethers in the urine of rubber workers. Although the urinary thioether assay appears useful in assessing the exposure of large numbers of people, the relatively high background-values which were found in both studies, limit its possibilities as a method for biological monitoring. Undoubtedly, cigarette smoking greatly attributes to the basic exposure of people to potentially alkylating chemicals. Cigarette smoke contains more than two thousand different compounds, including several known carcinogens and mutagens. There are many reports dealing with the carcinogenicity of cigarette smoking (Wynder, 1968; van Duuren, 1968, 1971; Bock, 1971). Recently, Yamasaki and Ames (1977) developed a method for concentrating mutagens from urine and demonstrated that smokers have mutagenic urine while nonsmokers do not. They proposed that this procedure be used in monitoring the urine of people or animals in toxicological studies.

In the present study mutagenicity and thioether excretions in urine of smokers and nonsmokers have been compared. An improved analytical procedure for the estimation of thioethers enabled us to evaluate the interference of cigarette smoking in the thioether test.

Materials and Methods

Chemicals

Amberlite, type XAD-2, was obtained from Serva (Heidelberg, FRG). Nicotinamide adenine dinucleotide phosphate (NADP) disodium salt, glucose-6-phosphate disodium salt and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were obtained from Boehringer (Mannheim, FRG), D-biotin and L-histidine-HCl from Sigma (St. Louis, USA) and citric acid monohydrate from J. T. Baker Chemicals (Deventer, the Netherlands). Purified agar, nutrient broth and yeast extract were purchased from Difco Laboratories (Detroit, USA), D-glucose, metaphosphoric acid [$(HPO_3)n$], sodium borohydride ($NaBH_4$) and N-acetyl-L-cystein from Merck (Darmstadt, FRG).

Bacterial Strain

Salmonella typhimurium TA 1538 was used throughout this investigation. This strain was a gift from Dr. B. N. Ames.

Microsomal Enzymes

Nine thousand g supernatant derived from liver homogenates of rats pretreated with Aroclor 1254 was purchased from Bionetics (Kensington, USA).

Urine Samples

Samples of urine from male smokers and nonsmokers being on normal diets and not receiving any medication were collected from 2.00—6.00 p. m. The subjects were classified in four groups

as follows: nonsmokers, persons smoking 1—10, 11—20, and 20 or more cigarettes a day ($n = 5$ for each group).

In a separate experiment urine samples of one of the subjects were collected daily from 10.00 a.m. until 6.00 p.m. for several days. During this period he smoked a number of cigarettes varying for 0—20 daily. Urine samples were frozen immediately and stored without preservatives at −20° C until experimental assay.

Assay of Creatinine

The creatinine concentration of each urine sample was determined as described by Gorter and De Graaff (1955).

Mutagenicity Testing

The method for concentrating mutagens and determining mutagenicity in urine samples has been described by Yamasaki and Ames (1977). We modified this method by using urine concentrates obtained from a volume of urine corresponding to an amount of 1.0 mmol creatinine instead of urine concentrates from 100 ml portions. The urine was put through a column with a 4 cm^3 bed volume of Amberlite XAD-2 and the adsorbed material was eluted with 10 ml acetone. The eluate was evaporated to dryness and the residue was dissolved in 0.4 ml dimethyl sulfoxide. 0.1 ml of this solution was added per plate and assayed for mutagenicity with the S. typhimurium tester strain TA 1538 in the presence of the microsomal enzymes as mentioned above. Urinary mutagenicity was expressed as the number of revertants per mmol creatinine per 10^6 bacteria. About 10^8 bacteria were plated in each experiment. It was found that in no case more than 15% of the bacteria were killed by the urine concentrate.

Assay of Urinary Thioether Concentration

The general procedure for the determination of urinary thioethers has been described previously (Seutter-Berlage et al., 1977). In the present study improved results were obtained by applying acid extraction and reduction of the samples prior to alkaline hydrolysis.

To a 5.0 ml sample of urine in a glass-stoppered tube 0.125 ml of 4 N HCl was added. Then 10 ml ethyl acetate was added and the urine was extracted by shaking vigourously several times and allowing the layers to separate. The extraction was repeated with 10 ml ethyl acetate. The ethyl acetate layers were collected, evaporated to dryness and subsequently dissolved in 2.5 ml water.

A 1.0 ml aliquot of this solution was pipetted in a test tube (brown glass, stoppered) and 0.10 ml anti-foam reagent, consisting of 90% ethanol and 10% octanol, was added, followed by the addition of 1.0 ml 5% $NaBH_4$. The tube was incubated unstoppered in a waterbath of 60° C for 15 min in order to reduce all disulfide compounds to mercaptans. Immediately after incubation the solution was neutralized with 0.50 ml 2.7 N HCl. After 10 min 0.20 ml of a metaphosphoric acid solution (10 mg/ml) was added. Five min later the sulfhydryl concentration of the mixture was determined according to Ellman (1959) as follows.

A 0.25 ml aliquot of the incubation mixture was added to 2.3 ml of a freshly prepared mixture consisting of 2.0 ml 0.5 M Sörensen-phosphate buffer (pH 7.1) and 0.3 ml DTNB solution (0.4 mg DTNB per ml 1% sodium citrate solution). Absorbances were read at 412 nm. Corrections were made for the attribution to the absorbance by the colour of the urine extract and of the DTNB solution. N-acetyl-L-cystein was used as a reference standard.

1.0 ml 4N NaOH was added to the rest of the incubation mixture. The solution was saturated with N_2, the tubes were closed and alkaline hydrolysis was performed at 100° C for 50 min in order to hydrolyze the thioether compounds. After incubation the tubes were cooled in ince for 10 min and neutralized with 1.0 ml 4N HCl. The sulfhydryl concentration was determined as described above.

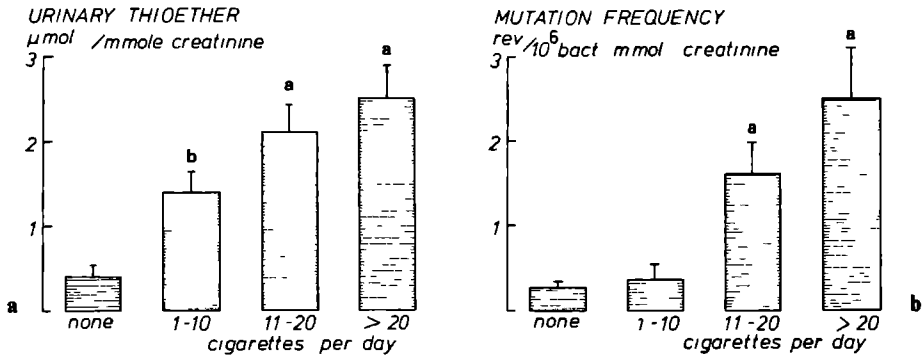

Fig. 1. **a** Thioether concentrations of urine samples from smokers and nonsmokers. Thioethers were assayed as described in the Materials and Methods section and are expressed as µmol SH-/mmol creatinine. Values are presented as means ± S.E.M. Each group consisted of five subjects. **b** Mutagenicity of urine from smokers and nonsmokers. The mutagenic activity was determined as described in the Materials and Methods section and expressed as the number of revertant colonies of S. typhimurium TA 1538 per mmol creatinine per 10^6 bacteria. Mean values ± S.E.M. are presented. Each group consisted of five persons. **a** Significantly different from the preceeding group at $P < 0.05$ (Wilcoxon, two-sample test). **b** Significantly different from the preceding group at $P < 0.01$ (Wilcoxon, two-sample test)

The differences in absorbance that were measured before and after alkaline hydrolysis represent the concentration of thioether compounds. The urinary levels of thioether compounds were expressed as µmol SH-/mmol creatinine.

Results

Urinary Concentrations of Thioether

As a consequence of the improved analytical procedure, which allowed us to differentiate between disulfides and thioether compounds, substantially lower normal thioether levels in human urine were found, as compared with those in previous studies (Seutter-Berlage et al., 1977; Vainio et al., 1978). As is demonstrated in Fig. 1, it was possible to distinguish between nonsmokers and the different categories of smokers at the significance level even with a small group of subjects. An increase in the number of cigarettes smoked a day was reflected by an increased urinary thioether excretion.

Excretion of Mutagens in Urine

The concentration of mutagenic agents in the urine of cigarette smokers was expressed as mutation frequency, i.e., the number of revertant colonies per million bacteria, per mmol urinary creatinine.

In Fig. 1b the mutation frequencies in urines from the different smoking groups are compared. Persons smoking up to 10 cigarettes a day did not show a significant increase in the mutation frequency when compared with nonsmokers. Urine mutagenicity in the group of persons smoking 11—20 cigarettes a day and

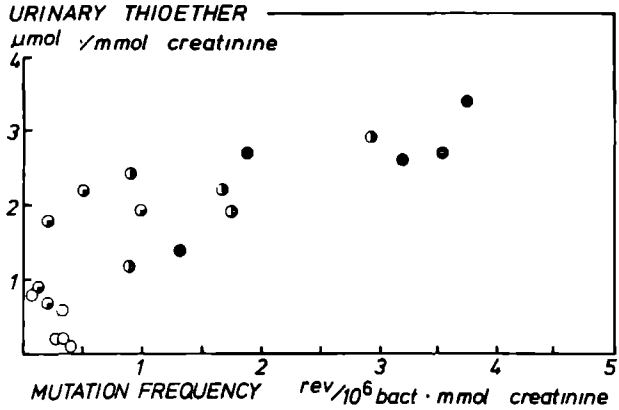

Fig. 2. Comparison between the urinary thioether excretion and mutagenicity of urine from the different smokers and nonsmokers. ○: nonsmokers; ◔: 1—10 cigarettes/day; ◑: 11—20 cigarettes/day; ● more than 20 cigarettes/day

in the group of persons smoking more than 20 cigarettes daily significantly increased when compared with the preceding group.

Relation Between the Excretion of Thioethers and Mutagens

In Fig. 2 the urinary thioether excretion is plotted as a function of the occurrence of potentially mutagenic agents in urine. It can be seen that smokers excrete more thioether compounds and show a higher mutation frequency in the urine when compared with nonsmokers.

Smoking up to about 10 cigarettes a day caused no statistically significant change in the mutation frequency whereas the urinary thioether excretion shows a remarkable increase. On the other hand, for the heavy smokers it appeared that the urinary mutation frequency increased strongly, while there was hardly any increase in the urinary thioether excretion.

Kinetics of Urinary Excretion of Mutagens and Thioethers

The time-dependent course of the urinary excretion of thioethers and mutagens, due to cigarette smoking, was studied by monitoring one single subject during a period of 20 days. The daily amount of cigarettes gradually increased from 0 to 20 cigarettes. The experiment ended with a non-smoking period of four days. The results are shown in Fig. 3. It can be seen that the appearance in the urine of the mutagens that are inhaled during smoking and excreted unchanged or as metabolites, is fairly well related to the number of cigarettes smoked during that day. This is demonstrated, e.g., at the end of the smoking period where no smoking immediately resulted in a rapid decrease in urinary mutagenicity. On the other hand, the excretion of thioethers responded more slowly to a change in the number of cigarettes smoked.

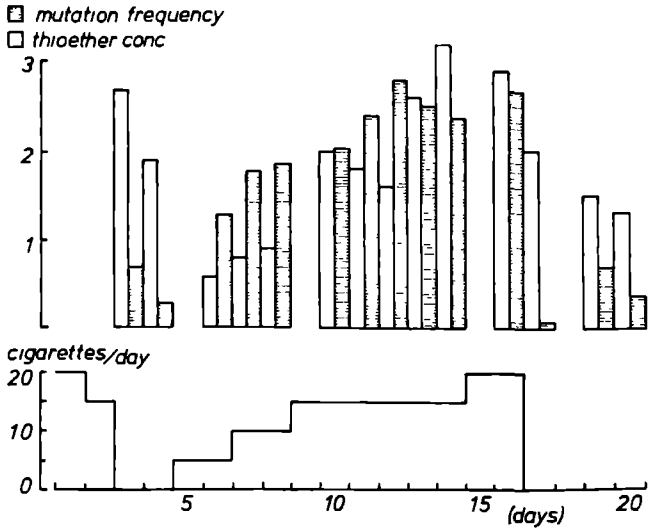

Fig. 3. Mutagenic activity detected with S. typhimurium TA 1538 and thioether concentrations of urine samples from one single subject during a period of 20 days. Mutagenicity and thioether concentration are expressed as revertants per 10^6 bacteria per mmol creatinine, and µmol SH-/mmol creatinine, respectively. Note that the urinary mutagenicity and thioether concentrations follow the variations in smoking pattern

Discussion

The analytical procedure for the assay of urinary thioether concentrations used in this study, showed much lower background values than those found in previous studies (Seutter-Berlage et al., 1977; Pentz, 1978; Vainio et al., 1978). This is mainly due to the reduction of the urine samples with $NaBH_4$ before the alkaline hydrolysis of the thioethers, which enabled us to make corrections for the contribution of urinary disulfides to the amount of sulfhydryl groups finally measured. This is in particular important for the elimination of the interference by the disulfide cystine, which normally is present in urine at an average concentration of 49 µmol SH-/mmol creatinine (ranging from 21 to 131) (Diem, 1960). In addition, the present thioether test has been made more selective by the extraction of acidified urine samples with ethyl acetate. The values obtained represent the excretion of acidic, sulfur-containing compounds, most probably detoxication products such as mercapturic acids, and thus may reflect the exposure to potentially alkylating agents.

In applying this method, we were able to differentiate between the urinary thioether values of smokers and nonsmokers, and even between those of light, moderate and heavy smokers (Fig. 1a).

Another technique that has been proposed to be useful in monitoring people for exposure to high risk substances, is the detection of mutagenicity in the urine (Durston and Ames, 1974; Legator et al., 1975; Yamasaki and Ames, 1977). We think that mutagenic testing of urine might be particularly valuable in diagnosing

exposures, in which the hazardous compounds are inadequately detoxified by the organism, or, otherwise, can be re-activated after initial detoxication. This test could therefore be complementary to the thioether test, because the value of the latter mainly depends on an effective detoxication, namely through the conjugation of reactive metabolites with SH-groups.

We found mutagens in the urine of smokers, especially in the urine of heavy smokers. Most of the excreted mutagens need metabolic activation, since mutation frequencies were much lower in the absence of microsomal enzymes. These findings are in accordance with the results reported by Yamasaki and Ames (1977). We suggest, however, that more accurate quantification of the urinary mutagenicity can be made by taking into account the creatinine concentration of urine. The variance in the values of both urinary thioether and mutagenicity, as clearly shown in Fig. 2, may be partly the result of individual differences in smoking habits (depth of inhalation, filter cigarettes, length of the discarded butt, etc.).

The absence of a significant increase in mutagenicity of the afternoon urine of persons smoking up to 10 cigarettes a day (Fig. 1b), might be due to the fact that these light smokers generally smoked during the evening of the day before. Possibly, the greater part of the potential mutagens that were inhaled during smoking had already been eliminated. This is supported by the data of Fig. 3 showing that after cigarette smoking mutagens appear much faster in the urine than the thioether products. This phenomenon also excludes the possibility that the thioethers excreted in the urine have mutagenic properties.

The combination of the thioether and mutagenicity tests as presented here may be valuable in monitoring people who are exposed to occupational levels of potentially alkylating agents. The present data indicate that cigarette smoking may interfere with the test results.

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EXCRETION OF MUTAGENS IN HUMAN URINE AFTER PASSIVE SMOKING

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SUMMARY

Eight non-smokers were experimentally exposed to cigarette smoke by staying in a poorly ventilated room together with heavy smokers for 6 h. Air samples were taken and the extract appeared to contain mutagenic substances. This is in accordance with the presence of carcinogens in tobacco smoke. Inhalation of the contaminated air by the passive smokers resulted in an increase in the urinary excretion of products mutagenic in the *Salmonella/* microsome assay. This observation suggests that there is a causality in the association between increased cancer risk and passive smoking, as was found by other investigators.

INTRODUCTION

It is a question of far-reaching significance, whether passive inhalation of tobacco smoke has only annoying, reversible effects like irritation of the eyes and the respiratory tract, or can cause permanent injury to health. Does passive smoking on the long run lead for example to an increased risk of cancer?

In 1981 Trichopoulos et al. [13] reported results from a case-control study demonstrating a statistically significant association between husbands smoking and women's cancer risk. About the same time, Hirayama [8] came to the same conclusion on the basis of a more extensive epidemiological investigation. He stated that passive and involuntary exposure to cigarette smoke markedly increases the risk of lung cancer. Contrary to these reports Garfinkel [7] failed to show such a statistically significant relationship.

Recently, the discrepancies between the results of these epidemiological studies have been discussed at length [10,11]. In particular, objections were

made to the quantitative interpretations with respect to the increased risk of lung cancer in passively smoking women.

In the present investigation an attempt was made to study the causality of an increased cancer risk due to passive smoking. Thereto, urine samples of passive smokers staying in a smoky room for 6 h were screened for the presence of mutagens. Measurement of urinary mutagenicity has been used successfully to establish environmental exposure to mutagenic or carcinogenic agents. In previous studies it was shown that active cigarette-smoking leads to the appearance of mutagens in the urine [4,17]. Other investigations are dealing with urinary mutagenicity after occupational or therapeutic exposure to carcinogenic substances [2,5,12].

MATERIALS AND METHODS

Collection of urine samples

Urine samples were collected from 8 male non-smokers and 10 smokers of either sex. The age of the subjects ranged from 25 to 35 years. It was assured that the non-smokers did not use any drugs. The non-smokers together with the heavy smokers stayed in a room (110 m^3) with poor ventilation for 6 h. During this period 157 cigarettes were smoked by the smokers. Just before the exposure was started, urine was collected and discarded. Thereupon urine was collected for a 12-h period. In addition urine of non-smokers was collected for the same period the day before and the day after the exposure to cigarette smoke. After collection, the urine samples were stored at −20°C until assayed. All samples were handled at one go to avoid negative influences of variations in the assay on the final result.

Method for concentrating mutagens present in urine

Thirty percent of a 12-h urine sample was loaded on an amberlite XAD-2 column with a 4-cm^3 bed volume. The column was washed 3 times with 5 ml of aqua dest. The adsorbed material was eluted with 10 ml of acetone. The eluate was evaporated to dryness under nitrogen at 60°C and the residue was dissolved in 0.45 ml of dimethylsulphoxide.

Air sampling

During the stay of persons in the room filled with smoke, a total of about 500 ℓ of smoky air was bubbled (1.3 ℓ/min) through 2 cylinders (in series) filled with ice-cold hexane. The same was done 1 day before and 1 day after the experiment, when no smokers were present in this room. The hexane portions were combined and evaporated to dryness, using a rotary evaporator. The final residue was taken up in 1 ml of dimethylsulphoxide.

Salmonella/*microsome assay*

Urine and air concentrates (0.1 ml per plate) were assayed for mutagenicity with the *Salmonella typhimurium* tester strain TA1538 in the presence of

S9 mix [1]. Every determination was done in triplicate. After incubation of the plates for 48 h at 37°C the number of revertant colonies was counted.

S9 mix

The S9 mix was prepared according to Ames et al. [1] and contained per ml, 100 μl of a hepatic 9000 × g supernatant from Aroclor 1254 induced rats and a NADPH-generating system.

Statistical analysis

The experimental data (8 cases (i = 1,2,..,8); 3 observations x_{ij} (j = 1,2,3)) were handled as follows. In our particular case we formulated the nullhypothesis: H_0: per case there is no preference for the highest value in any of the 3 observations; and the alternative hypothesis, H_1: per case there is a preference for the highest value in the second observation. This results in a binomial test with probability of success 0.33 if H_0 is true. "Success" is defined as "the value of the second observation being the highest".

RESULTS

Mutagenicity in urine

Urine samples of 8 non-smoking persons were collected before, during and after the passive exposure to cigarette smoke and mutagenicities were measured. The results are shown in Table 1. For 6 out of 8 persons highest

TABLE 1

MUTAGENICITY OF URINE FROM PASSIVE SMOKERS

Non-smoker	R_T/R_S-value[a]		
	Collection of urine samples[b]		
	Day before passive smoking	Day of passive smoking	Day after passive smoking
a	1.8	5.5	3.1
b	3.4	4.2	2.3
c	2.9	3.4	2.3
d	3.1	3.0	2.4
e	3.8	2.7	2.2
f	3.1	4.2	3.2
g	2.4	3.1	1.8
h	4.0	5.1	2.9

[a] R_T/R_S = Mean no. of revertants found with urine/Mean no. of revertants found with aqua dest.

[b] Detailed information is given in Materials and Methods.

values were observed at the day of passive smoking. Statistical analysis of the data revealed a significant enhancement of urinary mutagenicity during passive smoking ($P = 0.02$).

Urine of the 10 smokers was collected the day of the experiment and handled in the same way as the urine of the passive smokers. Relative urinary mutagenicity values (R_T/R_S) ranged from 9.0 to 42.0 with a median value of 23.0.

Mutagenicity in the air

During the passive smoking experiment the ambient air in the room, gradually contaminated with cigarette smoke, was tested for the presence of mutagens. A concentrate equivalent to 50 ℓ of air, containing the cigarette smoke, revealed 155 revertant colonies per plate. Testing a concentrate of an equal volume of air from the same room the day before or the day after, when no smokers were present, revealed 12 and 16 revertant colonies per plate, respectively.

DISCUSSION

These results clearly indicate that during experimental conditions, simulating passive smoking, compounds are inhaled that lead to urinary excretion of products which are mutagenic in the *Salmonella*/microsome assay. This finding is in good agreement with the observation that the ambient air in the smoky room contained a substantial amount of mutagenic substances as compared with the background as measured the day before and the day after the experiment. The mutagenicity in the air sample is in conformity with the data about the presence of carcinogens in tobacco smoke. In 1964 Galuskinova [6] reported the presence of benzo[*a*]pyrene in the smoky atmosphere of social meeting rooms and restaurants. Other investigators found that the amount of some carcinogens, e.g. β-naphthylamine, in sidestream smoke is many times that in mainstream smoke [14].

Since it is generally acknowledged that most of the genotoxic carcinogens can be detected by in vitro mutagenicity tests, our results about the mutagenicity in urine of passive smokers can be considered as an indication of carcinogenic exposure. This is in support of the reports of Hirayama [8] and of Trichopoulos et al. [13], who suggested a statistically significant association between husbands smoking and women's cancer risk. Nevertheless it can be doubted whether the estimations of the relative risks due to passive smoking, as made by these authors, are realistic [11].

It is known that the incidence of lung cancer is well-correlated with the smoking habits of the people concerned [3,16]. In a previous paper we showed that there exists a relationship between the mutagenicity in urine and the number of cigarettes smoked [4]. It is an interesting question, whether urinary mutagenicity might give in some way a reflection of the relative cancer risk of smoking.

In the urine of 8 non-smokers we found a median urinary mutagenicity value (R_T/R_S) of 3.7 during the experimental passive smoking. The median urinary mutagenicity value of these persons on the day before and after the passive-smoking experiment was 2.8. In urine of the 10 heavy smokers (> 20 cigarettes/day) we found urinary mutagenicity values ranging from 9.0 to 4 2 with a median value of 23.0. Thus the increase in urinary mutagenicity due to the passive exposure to cigarette smoke is about 4% of the increase observed with the active smokers during the experiment. Judging from these data, it is very tempting, though speculative, to suppose that the increased risk of cancer for passive smokers under comparable circumstances is of the same order of magnitude.

During the last decade society has become very concerned about the environmental cancer risk. Inhalation of air-pollutants, like PAHs produced in the combustion of, for instance, coal and oil, has been considered as a major cause of the increased pulmonary cancer [15]. For smokers the risk is multiplied, because the presence of many other carcinogenic substances in the cigarette smoke. It is important to note that the benzo[*a*]pyrene concentration of cigarette smoke is 500--1000 times higher than the average value in the city air [15]. In addition, cigarette smoke may contain inducers of microsomal oxidation by which other pre-carcinogenic substances are converted into their active forms [2,9].

The experimental results of the present study and the available epidemiological data suggest that non-smokers involuntarily inhale potential carcinogens. Strategies to control the environmental cancer problem can be only successful if the health-hazards of passive smoking are sufficiently acknowledged.

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Chapter 6

Mutagenicity of urine from nurses handling cytostatic drugs, Influence of smoking

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Mutagenicity of Urine from Nurses Handling Cytostatic Drugs, Influence of Smoking*

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Summary. Mutagenicity towards *Salmonella typhimurium* TA 100 of urine from smoking nurses, who were occupationally involved in the treatment of patients with cytostatic drugs, was significantly increased in comparison with that of smoking control subjects Mutagenicity towards *Salmonella typhimurium* TA 100 was not increased in exposed non-smokers when compared to control non-smokers. In smoking subjects urinary mutagenicity appeared increased towards *Salmonella typhimurium* TA 1538 in the presence of S-9 mix.

Rats pretreated with Aroclor 1254 showed higher mutagenicity in their urine than untreated rats after cyclophosphamide administration. Therefore, the synergistic effect of smoking might be due in part to induction of enzymes involved in the mutagenic activation of cytostatic drugs Further, the animal experiments showed that cyclophosphamide (the most frequently used mutagenic cytostatic drug) can be absorbed after oral or percutaneous administration. Therefore, it is not excluded that differences in working hygiene between smokers and non-smokers also play a role.

Key words: Mutagens/urine – *Salmonella typhimurium* – Antineoplastic agents – Cyclophosphamide – Smoking – Environmental exposure

Introduction

Many cytostatic drugs used in anti-cancer therapy have been shown to be genotoxic in short-term assays (Benedict et al. 1977; Seino et al. 1978; Matheson et al. 1978). This genotoxicity in vitro is in most cases paralleled by carcinogenicity in animals (Harris 1976; Leopold et al 1979) For this reason these substances bring about a potential hazard to persons who are occupationally involved in the synthesis, formulation and administration of these drugs, e.g. during nursing of cancer patients. This risk has already been studied by some investigators, who showed an increase in sister chromatid exchanges and in chromosomal gaps in lymphocytes of nurses handling cytostatic drugs (Norppa et al. 1980; Waksvik et al. 1981).

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Treatment of patients or animals with these drugs resulted in the appearance of mutagens in their urine (Minnich et al. 1976; Pak et al. 1979; Balbinder et al. 1981). These mutagens can be detected for instance with the aid of the *Salmonella typhimurium* strains selected by Ames et al. (1975). Falck et al. (1979) detected mutagenicity in urine of non-smoking nurses working in oncology departments. The mutagenicity was reduced when special safety measures were applied (Falck et al. 1981).

It has been reported by Yamasaki and Ames (1977) and van Doorn et al. (1979) that smoking of cigarettes results in the excretion of mutagens in urine. In 1980 Falck et al. showed that urine from smoking rubber industry workers was much more mutagenic than urine from either non-smoking workers or smoking non-workers. This might be either a simple additional effect or an indication for a potentiating effect of smoking on urinary mutagenicity. The latter possibility is supported by the recent finding of co-mutagenic properties of urine concentrates from smoking people (Hannan et al. 1981). In the present study we investigated the combined effect of smoking and exposure to cytostatic drugs on the mutagenicity of urine from hospital workers involved in the preparation and administration of cytostatic drugs.

Materials and Methods

Chemicals

Amberlite, type XAD-2, was obtained from Serva (Heidelberg, FRG). Nicotinamide adenine dinucleotide phosphate (NADP) disodium salt, glucose-6-phosphate (G-6-P) disodium salt, D-biotin and L-histidine-HCl were obtained from Sigma (St. Louis, USA). Glucose and citric acid monohydrate were from Merck (Darmstadt, FRG) Purified agar was from Difco Laboratories (Detroit, USA), nutrient broth No. 2 from Oxoid Ltd. (Basingstoke, England) and cyclophosphamide from Asta (Brackwede, FRG). All other chemicals used were of highest purity obtainable.

Media

Media used in the bacterial mutagenicity experiments were prepared as described by Ames et al. (1975) We used Oxoid nutrient broth instead of Difco nutrient broth as suggested by Ames in a supplementary writing.

Cytostatic Drugs

Cytostatic drugs arrived at the wards in ampoules, vials or capsules, as dragées or as tablets. A 3-month survey of cytostatic drugs shows several substances that are mutagenic to *Salmonella typhimurium,* including doxorubicin, melphalan, lomustine, daunorubicin, cyclophosphamide, cis-platinum and mercaptopurine. During this period 114 g cyclophosphamide (252 dragées or flasks) and 38 5 g of the other mutagenic cytostatics (1138 ampoules, tablets, capsules or vials) were used.

Collection of Urine Samples

Urine samples were collected for 24 h from nurses ($n = 29$) and other people ($n = 3$) involved in the administration and preparation of cytostatics and from control persons ($n = 29$), mainly

secretaries, play leaders and receptionists, working on the same hospital wards. Falck et al. (1979) stated that urine should be collected after some working days. We started collection of the urine samples at the beginning of the third working day, mostly Wednesday. In this way the urine samples could be tested soon after collection. During sampling they were coded and stored at low temperatures After the collection of the urine samples they were stored at −20° C until assayed. As soon as possible the urine samples were tested by persons unacquainted with the origins of the urine samples.

In the animal experiments male Wistar rats, weighing about 250 g were used. The rats, having free access to water and food, were housed individually in stainless steel metabolism cages designed for the separate collection of urine and feces After administration of cyclophosphamide or doxorubicine, urine samples were collected for 24 h

Method for Concentrating Mutagens Present in Urine

Ten per cent of a 24-h urine sample was loaded on an amberlite XAD-2 column with a 4-cm^3 bed volume The column was washed three times with 5 ml of aqua dest. The adsorbed material was eluted with 10 ml of acetone The eluate was evaporated to dryness under nitrogen at 60° C and the residue was dissolved in 0.3 ml of dimethylsulphoxide (DMSO).

In one experiment 5% of a 24-h urine sample from a patient was loaded on an amberlite XAD-2 column The ultimate residue was dissolved in 0 3 ml of DMSO.

The total 24-h urine sample was loaded on a XAD-2 column in order to concentrate mutagens present in rat urine, the final residue was dissolved in 0 35 ml of DMSO

Solvent values (S) were obtained by using 1 l of aqua dest. instead of a 24-h urine sample

Salmonella Microsome Plate Incorporation Assay

Urine concentrate was added (0.1 ml per plate) and assayed for mutagenicity with the *Salmonella typhimurium* tester strain TA 100, and in a number of cases with the *Salmonella typhimurium* tester strain TA 1538 in the presence of S-9 mix Every determination was done at least in triplicate. After incubation of the plates for 48 h at 37° C the number of revertant colonies was counted.

S-9 Mix

The S-9 mix was prepared according to Ames et al. (1975) and contained per ml 100 µl of a hepatic 9000 *g* supernatant from Aroclor 1254 induced rats and a NADPH-generating system.

Results and Discussion

Mutagenicity in Urine from Patients and Rats Receiving Cytostatic Drugs

In Table 1 it is shown that rats treated with cyclophosphamide or doxorubicine have mutagenic urine. The urinary mutagenicity was detectable with the tester strain TA 100 without addition of S-9 mix.

Patients receiving cyclophosphamide also had urine which was mutagenic to the tester strain TA 100 without addition of S-9 mix (Table 2). It should be noted that urine from the patient receiving 400 mg a day appeared to be toxic to the bacteria after the concentration procedure mentioned in the materials and methods section. In this case only 5% instead of 10% of the 24-h urine volume was used.

The direct mutagenic properties of urine from the patients and rats that received cytostatic drugs are in accordance with the findings of several investigators, who demonstrated that many of these drugs have mutagenic properties per se or appear as direct mutagenic metabolites in urine, detectable with the *Salmonella typhimurium* strain TA 100 (Matheson et al. 1978; Pak et al. 1979).

Table 1. Mutagenicity of urine from rats treated with cytostatics

Mutagenicity towards TA 100 (his$^+$ revertants per plate)				
Urine[a] from rats treated[b] with			Urine[a] from untreated rat (control)	Aqua dest
	Cyclo-phosphamide	Doxo-rubicine		
	648	246	79	81
	637	213	71	78
	650	273	75	81
Mean	645	244	75	80
R_T/R_S-ratio[c]	8 1	3 1	0 94	

[a] A 24-h urine sample was put through a XAD-2 column The eluate was evaporated and the final residue was dissolved in 0 35 ml of DMSO, 0 1 ml of this solution was added per plate and assayed for mutagenicity with the *Salmonella typhimurium* strain TA 100 in the absence of S-9 mix

[b] Male Wistar rats weighing about 200 g were injected intraperitoneally with cyclophosphamide (100 mg/kg) or doxorubicine (15 mg/kg)

[c] $R_T/R_S = \frac{\text{Mean number of revertants found with urine}}{\text{Mean number of revertants found with aqua dest}}$

Table 2. Mutagenicity of urine from patients receiving cyclophosphamide

Mutagenicity towards TA 100 (his$^+$ revertants per plate)		
Urine[a] from patients receiving cyclophosphamide at a dose of		
	100 mg/day	400 mg/day[b]
	316	329
	321	354
	300	340
		342
R_T (mean)	312	341
R_S (mean)	66	50
R_T/R_S[c]	4 7	6 8

[a] Ten per cent of a 24-h urine sample was put through a XAD-2 column The eluate was evaporated and the final residue was dissolved in 0 3 ml of DMSO, 0 1 ml of this solution was added per plate and assayed for mutagenicity with the *Salmonella typhimurium* strain TA 100 in the absence of S-9 mix

[b] In this case only 5% of a 24-h urine sample was used

[c] $R_T/R_S = \frac{\text{Mean number of revertants found with urine}}{\text{Mean number of revertants found with aqua dest}}$

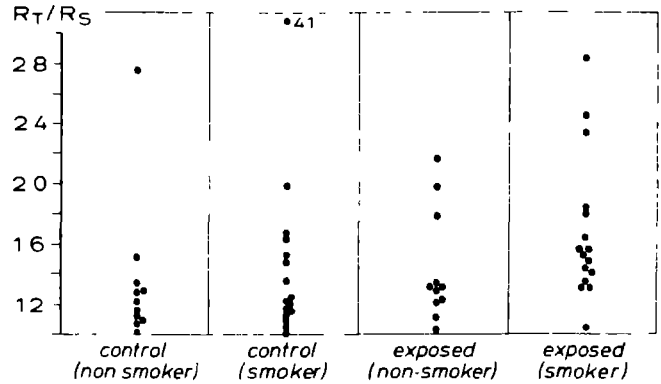

Fig. 1. Effect of exposure to cytostatics on urinary mutagenicity. The mutagenicity of urine samples from control subjects (non-smokers, smokers) and exposed subjects (non-smokers, smokers) as detected in the plate incorporation assay with the tester strain TA 100 in the absence of S-9 mix is expressed in R_T/R_S values. Median values are 1.23, 1.27, 1.34 and 1.55, respectively

$$R_T/R_S = \frac{\text{Mean number of revertants found with urine}}{\text{Mean number of revertants found with aqua dest.}}$$

Mutagenicity in Urine from Nurses and the Influence of Smoking

In Fig. 1 the mutagenicities of urine samples of exposed and control subjects are shown. Mutagenicity was determined with the aid of the *Salmonella typhimurium* strain TA 100 in the plate incorporation assay and given in R_T/R_S values. Both groups (exposed and control) are subdivided into smokers and non-smokers. The mutagenicity of urine from exposed smokers was significantly higher than that of the control smokers (Wilcoxon test: $P = 0.028$). In this respect exposed non-smokers did not differ from control non-smokers.

As has been reported earlier, smoking of cigarettes gives rise to the appearance of mutagens in urine. Testing of urine samples with the aid of the *Salmonella typhimurium* strain TA 1538 in the presence of S-9 mix is the method commonly used for detection of urinary mutagens as a consequence of smoking (Yamasaki and Ames 1977; van Doorn et al. 1979). The mutagenicity of urine from smoking subjects tested in this way is shown in Fig. 2. Mutagenicity of urine from smokers was significantly increased (Wilcoxon test: $P < 0.0001$).

The finding that urinary mutagenicity of exposed smokers as detected with TA 100 is higher than that of control smokers (Fig. 1) cannot be due to a difference in smoking behaviour. First, there is a good correlation between the number of cigarettes smoked and the urinary mutagenicity as detected with the strain TA 1538 in the presence of S-9 mix (van Doorn et al. 1979). From this and from Fig. 2 we may conclude that the group of control smokers did not consist of people smoking less than people of the group of exposed smokers. On the basis of the data obtained with TA 1538 it might be concluded that the group of control smokers even contains more heavy smokers. Second, a comparison of urinary mutagenicity of control smokers with that of the control non-smokers as detected with TA 100 (Fig. 1) did not give a statistically significant difference between the two groups

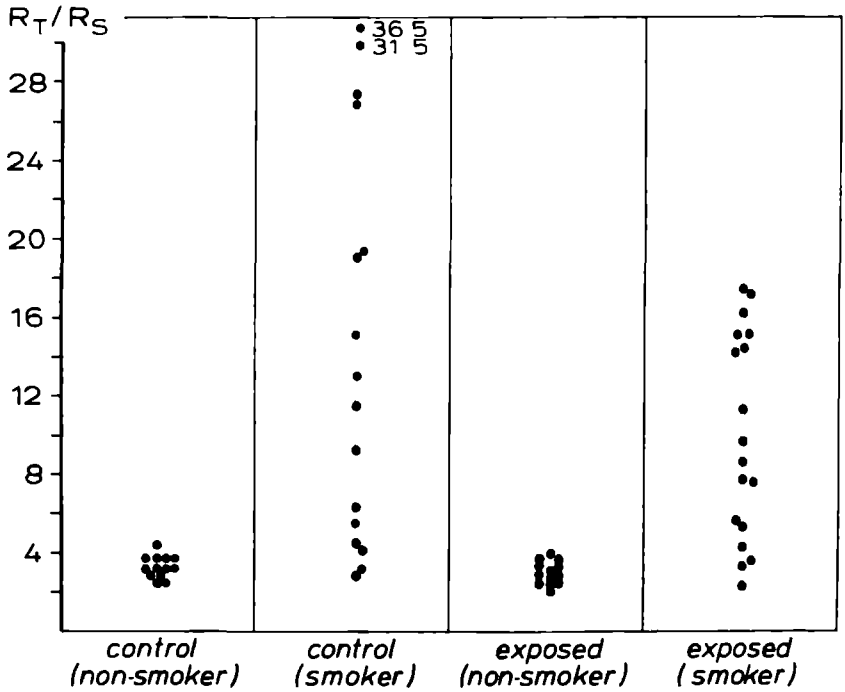

Fig. 2. Effect of smoking on urinary mutagenicity. The mutagenicity of urine samples from control subjects (non-smokers, smokers) and exposed subjects (non-smokers, smokers) as detected in the plate incorporation assay with the tester strain TA 1538 in the presence of S-9 mix is expressed in R_T/R_S values. Median values are 3.4, 12.5, 3.1 and 9.0, respectively

$$R_T/R_S = \frac{\text{Mean number of revertants found with urine}}{\text{Mean number of revertants found with aqua dest.}}$$

(Wilcoxon test: $P > 0.05$). Mutagens in urine as a consequence of smoking could not be detected with TA 100.

Synergistic Effect of Smoking

The increase in mutagenicity of urine from smokers exposed to cytostatics in comparison with the mutagenicity of urine from control smokers as detected with the strain TA 100 suggests a synergistic effect. Synergistic effects of smoking on the urinary mutagenicity have been observed by other investigators. Falck et al. (1980) showed a much higher mutagenicity in the urine from smokers working in the rubber industry than in urine from non-smoking workers or in urine from control smokers. Recently Wheeler et al. (1981) reported that smoking may have a synergistic effect on the appearance of mutagens in urine of coal-tar-treated psoriatic patients. It was found by Hannan et al. (1981) that cigarette smoke condensate and urine concentrate from smokers exhibit a co-mutagenic action on the mutagenicity of 2-aminoanthracene. The co-mutagenic factor was absent in urine concentrates from non-smokers.

Table 3. Mutagenicity of urine from a cyclophosphamide-treated patient mixed with urine from smokers and non-smokers

Urine concentrate[a] added to the topagar	Number of his$^+$ revertants/plate[b] (strain TA 100)
Patient	341 ± 5
Patient + smoker 1	170 ± 17
Patient + smoker 2	248 ± 16
Patient + non-smoker	194 ± 3
Smoker 1	142 ± 6
Smoker 2	156 ± 13
Non-smoker	137 ± 3

[a] Five per cent of 24-h urine samples from a patient, a smoker or from a non-smoker alone or combined was loaded on a XAD-2 column. The eluate was evaporated and the final residue was dissolved in 0.3 ml of DMSO; 0.1 ml of this solution was added per plate in quadruplicate and assayed for mutagenicity with the *Salmonella typhimurium* strain TA 100 in the absence of S-9 mix

[b] Mean values (± SEM) of four measurements of the same urine

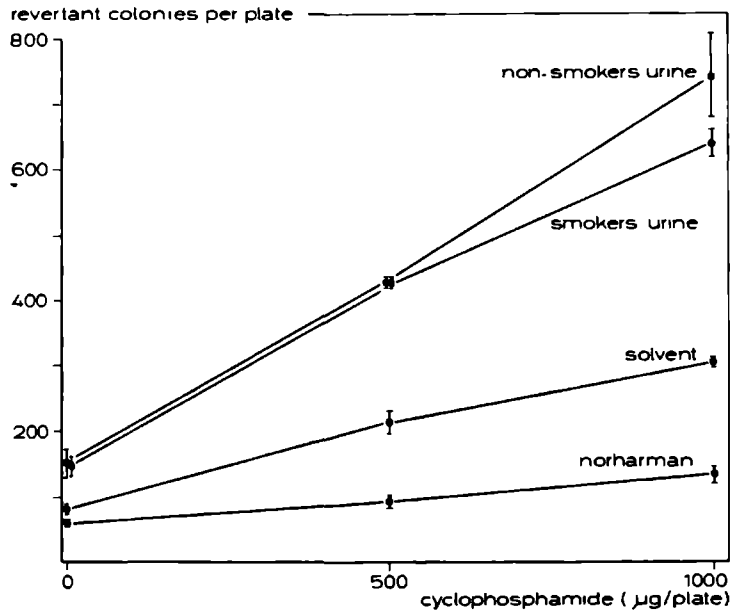

Fig. 3. Influence of urine concentrate on mutagenicity of cyclophosphamide. The mutagenicity of cyclophosphamide was determined in the plate incorporation assay, using the *Salmonella typhimurium* strain TA 100 in the presence of S-9 mix; 0.1 ml of urine concentrate from non-smokers or smokers, norharman (400 µg/plate) or the vehicle (DMSO), respectively, was added to the topagar

Treatment[a]	Mutagenicity towards TA 100 (his$^+$ revertants/plate)[b]	
	Urine[c] from	
Cyclophosphamide	Unpretreated rats	Aroclor 1254 pretreated rats[d]
Experiment 1	228 ± 9	338 ± 6
Experiment 2	245 ± 14	419 ± 13
Experiment 3	250 ± 7	424 ± 2
Saline	106 ± 8	91 ± 7

[a] Male Wistar rats weighing about 250 g were injected intraperitoneally with cyclophosphamide (20 mg/kg) or saline

[b] Mean values (± SEM) of three measurements of the same urine

[c] A 24-h urine sample was put through a XAD-2 column. The eluate was evaporated and the final residue was dissolved in 0.35 ml of DMSO; 0.1 ml of this solution was added per plate and assayed for mutagenicity with the *Salmonella typhimurium* strain TA 100 in the absence of S-9 mix

[d] Rats received a single dose of Aroclor 1254 (500 mg/kg) 5 days before the administration of cyclophosphamide

Table 4. Influence of Aroclor 1254 on the urinary mutagenicity of rats given cyclophosphamide

Because we only found an increased mutagenicity in urine from subjects exposed simultaneously to cigarette smoke and cytostatic drugs, the question arose whether in this case a co-mutagenic action of smoking was involved. To study a co-mutagenic effect of smoking on the urinary mutagenicity after exposure to cyclophosphamide, we combined the urine of a cyclophosphamide-treated patient with urine from smokers and a non-smoker and compared the urinary mutagenicity. The data presented in Table 3 show that neither urine from a non-smoker nor urine from a smoker increases the mutagenicity of urine from a patient receiving cyclophosphamide. It may be concluded from this that no direct co-mutagenic action is involved.

Another possibility is that the co-mutagenic effect takes place at the level of the mutagenic activation of cyclophosphamide. We studied the influence of urine concentrates from smokers and non-smokers and of the known co-mutagen norharman on the in vitro mutagenicity of cyclophosphamide tested with TA 100 in the presence of S-9 mix. The results are shown in Fig. 3. There was no difference in the increase in mutagenicity of cyclophosphamide caused by urine concentrate from smokers or non-smokers. This increase might be explained partly by the presence in urine concentrate of certain factors promoting bacterial growth, and not by the presence of specific co-mutagenic factors. Figure 3 even shows a decreasing effect of norharman, a known co-mutagenic substance present in cigarette smoke (Nagao et al. 1977).

Several compounds (3-methylcholanthrene, Aroclor 1254, and phenobarbital) are known to induce enzymes of the microsomal mono-oxygenase system. Jusko

Table 5. Mutagenicity of urine from rats after administration percutaneously or orally of cyclophosphamide

Treatment	Mutagenicity towards TA 100 (his$^+$ revertants per plate)[a]	
	Administration[b]	
Cyclophosphamide	On the skin	Orally
Experiment 1	198 ± 6	490 ± 16
Experiment 2	282 ± 8	532 ± 25
Experiment 3	304 ± 15	555 ± 8
Olive oil	117 ± 8	

[a] A 24-h urine sample was put through a XAD-2 column. After evaporation of the eluate, the final residue was dissolved in 0.35 ml of DMSO; 0.1 ml of this solution was added per plate and assayed for mutagenicity with the *Salmonella typhimurium* strain TA 100 in the absence of S-9 mix. Mean values (±SEM) of three measurements of the same urine

[b] Male Wistar rats weighing about 250 g were given 10 mg of cyclophosphamide in suspension in olive oil (0.1 ml), either on the close-shaven skin in the neck or per os

(1979) and Boobis et al. (1979) found that cigarette smoking significantly increased mono-oxygenase activity and cytochrome P-448 content in human liver. An increased mono-oxygenase activity can also be held responsible for the increase in urinary mutagenicity after exposure of smokers to (pre) mutagenic cytostatics. The data from Table 4 clearly show that pretreatment of rats with Aroclor 1254 results in a twofold increase in urinary mutagenicity after exposure of rats to cyclophosphamide. This finding supports the concept that the higher urinary mutagenicity in exposed smokers in comparison with exposed non-smokers is due to induction of activating enzymes as a consequence of smoking.

On the other hand, it cannot be excluded that the synergistic effect of smoking is partly caused by differences in working hygiene between smokers and non-smokers. In this respect, although the nurses do not smoke during work periods, a finger-shunt effect might play a role.

Possibility of Exposure to Cytostatic Drugs

Most probably, absorption of the cytostatic drugs by the nurses handling these substances may occur via:

1) Skin contact with the drug in solution or as dust, or skin contact with urine from patients treated with these substances
2) Inhalation of the dust of the drug or aerosolized drug particles

This means that the manner in which the preparations are unpacked when they arrive from the pharmacy department, the way the nurses handle these preparations and the urine from treated patients, and the waste and spilling of the preparations are all very important factors in regard to their exposure.

It is known that many polar and non-polar toxicants can easily penetrate the skin. Because most genotoxic cytostatic preparations are water-soluble, we were interested in absorption of these drugs through the skin, because this could be very important with respect to the route of intake. Therefore, experiments were done in which the urinary mutagenicity was compared between rats having received cyclophosphamide percutaneously or orally (Table 5). At 24 h after application, urine from percutaneously treated rats showed about 50% of the mutagenicity of the urine from rats having received cyclophosphamide per os.

Conclusion

It has been emphasized by several authors that smoking may potentiate or add to the health effects of workplace exposures (NIOSH 1979; WHO 1981; Joosting 1981). In the present study it was found that smoking increased urinary mutagenicity detectable with TA 1538 (Fig. 2). On the other hand, with TA 100 it appeared that smoking has a potentiating effect on the mutagenicity of urine from nurses exposed to cytostatic drugs (Fig. 1). It might be concluded therefore that smoking nurses, handling cytostatic agents, have a greater occupational risk than their non-smoking colleagues.

It should be emphasized that all measures should be taken to protect smoking as well as non-smoking hospital employees from exposure to genotoxic cytostatic drugs. Rules for safe handling of cytostatic drugs are extensively described elsewhere (Yorkshire Regional Cancer Organisation 1980).

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Addendum

Several studies show a greater increase in the alkylating or genotoxic activity of cyclophosphamide in vitro after incubation with phenobarbital-induced liver fractions than after incubation with control or polycyclic hydrocarbon-induced liver fractions. (Sladek 1972, Hales 1980a, Hales 1980b, Hales 1981). It is known that phenobarbital and 3-methylcholanthrene induce different cytochromes of the monooxygenase system: cytochrome P450 and P448, respectively (Conney et al. 1973).

The influence of cigarette smoking on drug metabolism in man is consistent with the known inductive effects of P448 stimulators in animal systems (Jusko, 1979). It can be questioned now, whether induction of P448 may result in the excretion of more mutagens in urine after administration of cyclophosphamide. The data presented in table 4 show that pretreatment of rats with aroclor 1254 resulted in an increase in urinary mutagenicity after exposure of rats to cyclophosphamide. This may be expected since aroclor 1254, a mixture of polychlorinated biphenyls, should induce hemeproteins that can also be induced by phenobarbital and 3-methylcholanthrene (Ryan et al. 1977). To evaluate the role of P448 induction in the excretion of mutagens in urine after exposure to cyclophosphamide, we have pretreated rats with 3-methyl-cholanthrene and studied the urinary mutagenicity after the administration of cyclophosphamide. The results are shown in the table below. It can be seen that phenobarbital pretreatment as well as 3-methylcholanthrene pretreatment as well as pretreatment with aroclor 1254 resulted in a remarkable increase in urinary mutagenicity after exposure of rats to cyclophosphamide.

INFLUENCE OF DIFFERENT INDUCERS OF THE MONOOXYGENASE SYSTEM ON THE URINARY MUTAGENICITY OF RATS GIVEN CYCLOPHOSPHAMIDE

Treatment[a]	Mutagenicity towards TA100 (his^+ revertants/plate)[b]			
	Urine from[c]:			
Cyclophosphamide	unpretreated rats	3-methylcholanthrene pretreated rats[d]	phenobarbital pretreated rats[e]	aroclor 1254 pretreated rats[f]
experiment 1	125 ± 9	315 ± 19	424 ± 20	528 ± 44
experiment 2	216 ± 12	608 ± 22	428 ± 22	719 ± 5
experiment 3	262 ± 9	1240 ± 30	430 ± 7	1100 ± 30
saline	106 ± 1	102 ± 8	73 ± 3	93 ± 5

[a] Male Wistar rats weighing about 200 g were injected intraperitoneally with cyclophosphamide (25 mg/kg)

[b] Mean values (± SEM) of three measurements of the same urine

[c] A 24-h urine sample was put through a XAD-2 column. The eluate was evaporated and the final residue was dissolved in 0.35 ml of DMSO; 0.1 ml of this solution was added per plate and assayed for mutagenicity with the Salmonella typhimurium strain TA100 in the absence of S9 mix

[d] Rats received a single dose of 3-methylcholanthrene (80 mg/kg) 24 hrs before the administration of cyclophosphamide

[e] Rats received phenobarbital (75 mg/kg) 96, 72, 48 and 24 hrs before the administration of cyclophosphamide

[f] Rats received a single dose of aroclor 1254 (500 mg/kg) 5 days before the administration of cyclophosphamide

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Chapter 7

Exposure to mutagens of workers involved in the preservative treatment of wood with creosote

GENOTOXIC EXPOSURE OF WORKERS CREOSOTING WOOD

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ABSTRACT

In a small wood preserving industry spot samples were taken from contaminated surfaces at several places and tested for mutagenicity. The results suggest that the application of a wipe-test can give a first indication of occupational exposure to mutagenic and carcinogenic substances, particularly when exposure occurs more via skin contact than via inhalation.

One of the pesticide chemicals in use for the preservation of wood is the mutagenic creosote. It was found that mutagens appeared in urine of rats after intraperitoneal administration of creosote. In spite of these results, no increase in mutagenicity could be detected in urine of creosote-workers in relation to their work.

INTRODUCTION

Creosote is well known as one of the pesticide chemicals in use for wood preservation. It is a mixture of oils that are separated in the distillation of coal tar and consists principally of liquid and solid hydrocarbons. Because it is known that coal tar contains many polycyclic aromatic hydrocarbons, we were interested in the mutagenicity of creosote. Mutagenic properties of creosote were established with the Salmonella/microsome assay[1].

Because of its mutagenicity, this substance might bring about a potential hazard to persons who are occupationally involved in the preservative treatment of wood. Production workers in the wood preserving industry can be

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exposed to creosote not only by inhalation of vapours, but also through skin and eye contact. It is known that workers exposed to coal tar products are at a high cancer risk[2].

In the present study possibilities of detecting the exposure to creosote were explored. At several places in the work area contaminated surfaces were examined for the presence of mutagens. In addition, since it was found that exposure of Wistar rats to creosote under experimental conditions leads to the excretion of mutagenic products in urine, an attempt was made to monitor creosote workers by measurements of urinary mutagenicity.

MATERIALS AND METHODS

Chemicals

The creosote type P1 that was used was from Cindu Chemicals B.V. (Uithoorn, The Netherlands). Bacterial β-glucuronidase (type IX) was obtained from Sigma (St. Louis, U.S.A.). Amberlite, type XAD-2 was purchased from Serva (Heidelberg, FRG). Purified agar was from Difco Laboratories (Detroit, U.S.A.), nutrient broth No. 2 from Oxoid Ltd. (Basingstoke, England). All other chemicals used were of highest purity obtainable.

Impregnation process

The preservative impregnation with creosote is accomplished by a pressure method (full-cell process). After a charge of wood is taken into a cylinder, an initial vacuum is applied for a period of at least 15 min. At the end of this period the vessel is filled with creosote having a temperature of at least 70°C (mostly 85-100°C), still maintaining the vacuum. Next, the vacuum is released and pressure with a maximum of 10 kgf/cm^2 is applied to the system. Pressure is maintained until the required gross absorption of creosote has been achieved (maximal 3 hours). This value varies depending upon the species being treated. At the end of the pressure cycle, the pressure is reduced to atmospheric level, the preservative returned to storage, and the treated wood often subjected to a final vacuum to remove excess creosote oil from the surface of the stock. The vacuum is released, the door of the vessel opened, and the treated wood removed.

Workers

We have studied the possible exposure of three workers. One of them is the operator of the cylinder. The other two workers are moving the wood in and out of the cylinder. Especially the operator would be briefly exposed to creosote vapours when the cylinder door is opened after treatment. The workers can also be exposed to residual surface creosote through skin contact. Most of the time they are wearing gloves.

Mutagenicity testing

The mutagenicity test was performed according to Ames et al.[3] with *Salmonella typhimurium* TA98 and TA100. We used Oxoid nutrient broth instead of Difco nutrient broth. Rat liver S9 (9000 *g* supernatant) fractions were prepared from male Wistar rats, pretreated with aroclor 1254. S9 mix contained 0.1 ml S9 per ml. Every determination was done in triplicate. After incubation of the plates for 48 h at 37°C the number of revertant colonies was counted.

Urinary mutagenicity

RATS

Male Wistar rats weighing about 200 g were purchased from TNO (Rijswijk, The Netherlands). The animals were housed individually in stainless steel metabolism cages, designed for the separate collection of urine and feces. The rats had free access to water and food (Hope Farms, Woerden, The Netherlands). Creosote, dissolved in olive oil, was injected intraperitoneally. Control rats received only olive oil.

Urine samples were collected for 24 h and stored at -20°C until assayed. Before they were assayed, the individual samples were completed to 15 ml and sterilized by filtration through 0.2 µm membrane filters.

Mutagenicity of the urine samples was determined using the *Salmonella typhimurium* strains TA98 and TA100[3]. 0.1 ml of a fullgrown suspension of the bacteria ($\pm$ 2 x 10^9 bact./ml) was added to the topagar, containing 0.3 ml of diluted urine, and was supplied with an activating enzyme system. This activating enzyme system consisted of either, 0.5 ml S9 mix, or 0.1 ml of a sterile ß-glucuronidase solution (1500 U/ml), or a combination of both[4].

WORKERS

Urine samples from the workers were gathered during ten consecutive days,

including two free weekends. During this period two portions urine were collected dayly: one sample overnight and one sample between 10.00 a.m. and 4.00 p.m. The mutagenicity was determined according to van Doorn et al.[5].

Monitoring of contaminated surfaces

For the detection of contamination of work areas with mutagenic creosote we applied a method based on the method described by Simmon and Peirce[6]. 5 ml of acetone or alcohol were put on a surface and the solvent was mopped up with a Kleenex tissue. The tissue was extracted twice with 25 ml of acetone or alcohol. The acetone or alcohol was evaporated to dryness using a rotary evaporator. The total residue was dissolved in 5 ml of dimethylsulphoxide (DMSO). 0.1 ml of this solution was added per plate and assayed for mutagenicity with the *Salmonella typhimurium* tester strain TA98 in the presence of S9 mix. Every determination was done in triplicate. After 48 h of incubation of the plates at 37°C the number of revertant colonies was counted.

RESULTS

Mutagenicity of creosote

Table 1 shows the experimental results in which the mutagenicity of

TABLE 1

MUTAGENICITY OF CREOSOTE

Creosote (µg/plate)	Number of his^+ revertants per plate[a)] Strain	
	TA98	TA100
0	16 ± 4	69 ± 4
2	17 ± 1	87 ± 9
5	21 ± 2	99 ± 2
20	134 ± 9	284 ± 12
50	336 ± 7	598 ± 9

a) Mean values ± S.E.M. of determinations in triplicate

creosote was established. The mutagenicity was assayed using the *Salmonella typhimurium* strains TA98 and TA100 in the presence of S9 mix and expressed as number of revertant colonies per plate.

Detection of mutagenic substances in the work environment

The work environment was examined on the presence of mutagenic substances. Spot samples from contaminated surfaces at several places were taken and tested for mutagenicity. These mutagenicity data are presented in table 2.

TABLE 2

CONTAMINATION WITH MUTAGENIC SUBSTANCES OF SOME SPOTS IN THE WORK ENVIRONMENT[a)]

	Number of his^+ revertants/plate[b)]	
	Solvent used for cleaning	
Sample	acetone	alcohol
spot 1	510 ± 29	395 ± 8
spot 2	558 ± 52	77 ± 2
spot 3	435 ± 31	109 ± 5
spot 4	231 ± 7	373 ± 20
spot 5	181 ± 9	23 ± 1
negative control	45 ± 3	31 ± 2
positive control	c)	324 ± 8
Spontaneous	19 ± 2	

a) Experimental details were as mentioned in Materials and Methods.
b) Mean values (± S.E.M.) of 3 determinations on the same sample.
c) No value available because of a toxic effect on the bacteria.

Spots No. 1,2,3 and 4 were on metal surfaces. Spot 1 and 2 were on a grip of the door of the cylinder and on a handle very close to this door, resp. Spot 3 was on the banistairs about 3 m away from the cylinder. Spot 4 was on

a lorry used for the transport of wood in and out of the cylinder. Spot 5 was on wood that was not treated with creosote. This wood was piled up about 15 m away from the cylinder. A negative control was made from a surface of a clean table in our laboratory. A spot on the surface of creosoted wood served as a positive control. These results show that there is a contamination of the work environment with mutagenic substances.

It can be remarked that extraction with acetone reveals higher mutagenic values than the extraction with alcohol

Animal experiments

Administration of creosote to rats (250 mg/kg body weight, i.p.) resulted in the appearance of mutagens in urine detectable with the strains TA98 and TA100 (table 3). The highest mutagenicity values were detected after the

TABLE 3

MUTAGENICITY TOWARDS THE SALMONELLA TYPHIMURIUM STRAINS TA98 AND TA100 OF URINE FROM A CREOSOTE-TREATED RAT AFTER THE ADDITION OF DIFFERENT ENZYME PREPARATIONS

	Number of his^+ revertants/plate[a]			
	Treatment			
	Creosote[b]		Control	
Addition[c]	TA98	TA100	TA98	TA100
None	43 ± 2	188 ± 9	37 ± 1	175 ± 8
S9 mix	44 ± 5	202 ± 8	39 ± 4	151 ± 3
S9 mix + β-glucuronidase	123 ± 8	341 ± 10	43 ± 3	175 ± 12
β-glucuronidase	76 ± 11	275 ± 18	47 ± 2	166 ± 2

[a] Mean values (± S.E.M.) of 3 measurements of the same urine.
[b] Rats were injected i.p. with a dose of 250 mg/kg.
[c] For experimental details see Materials and Methods.
150 Units of β-glucuronidase were used.

addition of S9 mix in the presence of β-glucuronidase to the urine.

Absence of mutagenicity in urine of creosote workers

We were interested whether the workers involved in the preservative treatment of wood with creosote had mutagenic urine. We collected urine samples on 10 consecutive days. Using *Salmonella typhimurium* TA98 in the presence of S9 mix and β-glucuronidase, we failed to detect an increase in urinary mutagenicity that could be related to their work.

DISCUSSION

These results clearly show the presence of mutagenic substances in the work environment of a wood-preserving industry. Most likely this mutagenicity is due to the presence of creosote.

The method used for monitoring the work environment for the presence of mutagens is very easy to be done. Our procedure is principally based on the method of Simmon and Peirce[6] who introduced it to detect the spill of carcinogens and mutagens in laboratories. The present results suggest that the application in the work environment of this wipe-test could give a first indication of occupational exposure to mutagenic and carcinogenic substances. This might be important, particularly in those cases where exposure can occur rather via skin contact than via inhalation.

In spite of the substantial contamination of the work environment with mutagenic substances, no increase in mutagenicity in urine of the workers was detected in relation to their work. On the other hand it was found that mutagens appeared in urine of rats after intraperitoneal administration of creosote. Most probably the absence of mutagens in urine of creosote workers has to be attributed to (1) a relative low level of exposure, for instance owing to the wearing of protective gloves, (2) a wrong choice of the time of taking urine samples, (3) insensitivity of the urinary mutagenicity assay. Presumably more selective methods of biological monitoring may give more information about the internal exposure

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Addendum

In this chapter it is demonstrated that mutagens appeared in urine of rats after intraperitoneal administration of creosote. In this way it is shown that rats who are exposed internally to creosote have mutagens in their urine.

One of the main routes of entrance of creosote in workers might be via absorption through the skin. In relation to this it is interesting to know whether mutagens present in creosote can penetrate the skin. Wheeler et al. (1981) showed that coal-tar treated psoriatic patients have mutagenic urine. This indicates that certain mutagens present in coal tar can penetrate the human skin to be excreted via the urine in a later stage. Creosote, a product derived from coal tar, may contain the mutagenic substances present in coal tar which can penetrate the skin.

In an additional experiment (see table) we show that mutagens appeared in urine of rats after the administration of creosote on the skin. This means that mutagens present in creosote are able to pass through the skin of the rat. This observation and the finding of Wheeler et al. (1981) make it reasonable that human exposure to creosote via the skin in principle can result in mutagenicity in the urine. Whatever the reason might be, at any rate the absence of mutagenicity in the urine of creosote workers can not be explained by the inability of mutagens in creosote to penetrate the human skin.

Wheeler LA, Saperstein MD, Lowe NJ, Mutagenicity of urine from psoriatic patients undergoing treatment with coal tar and ultraviolet light. J. Invest. Dermatol. 1981; 77: 181-185.

MUTAGENICITY OF URINE FROM RATS AFTER ADMINISTRATION OF CREOSOTE P1 ON THE SKIN OR ORALLY

Treatment	Mutagenicity towards TA98 (his revertants per plate)[a]	
	Administration[b]	
Creosote P1	On the skin	orally
Experiment 1	89 ± 2	91 ± 5
Experiment 2	96 ± 7	104 ± 5
Experiment 3	—	118 ± 5
Spontaneous	27 ± 2	

[a] For experimental details see Materials and Methods. 0.3 ml of diluted raturine was tested for mutagenicity in the presence of S9 mix and β-glucuronidase (150 U). Mean values (± SEM) of three measurements of the same urine.

[b] Male Wistar rats weighing about 200 g received a dose of creosote P1 (250 mg/kg) in solution in olive oil (0.1 ml) either on the close-shaven skin in the neck or per os.

The ability of many chemicals to induce cancer is connected with their genotoxic properties. These properties imply that such compounds can react with DNA. Genotoxicity of chemicals can be detected in so-called short-term assays. Among these, the Salmonella/mutagenicity assay is one of the most evaluated and most reliable short-term assays.

In order to safeguard people effectively against the risk of cancer by chemicals, the knowledge of two kinds of data is of primary importance. First, one should have information about the intrinsic carcinogenic properties of chemical compounds. Secondly it is important that exposure to such chemicals can be recognized.

Both aspects have been discussed in this thesis. On the one hand, investigations are mentioned in which the Salmonella/mutagenicity assay was applied to detect genotoxic properties of chemicals; on the other hand possibilities to determine exposure to carcinogenic chemicals with the aid of this assay were studied.

PART 1 mainly deals with studies *in vitro* on the suitability of the Salmonella/mutagenicity assay, applied in several forms to detect mutagenic properties of chemical products.

With the aid of the Salmonella/microsome assay it was found that creosote P1 has mutagenic properties. This product is well known as a pesticide in use for wood preservation and was recently denoted as non-mutagenic in bacterial assays. The Salmonella/microsome assay was subsequently used in combination with chemico-analytical methods to characterize the mutagenic components of creosote. Concentrations of benzo(a)pyrene and benz(a)anthracene were found to be 0.18 and 1.1 per cent, respectively (chapter 1).

Often, electrophilic compounds or intermediates are able to react with endogenous glutathione. After finding a decrease in the concentration of liver glutathione due to the administration of toluene or xylenes, we wondered whether these compounds or their metabolites might have mutagenic properties on account of their electrophilicity. However, no mutagenicity was detected in the Salmonella/microsome assay of these compounds or some of the suspect metabolites. This means that electrophilicity does not

necessarily imply mutagenicity (chapter 2).

In view of the prediction of genotoxic activity *in vivo*, it is a relevant question to what extent the activity of the liver fraction, added in the Salmonella/microsome assay, reflects the biotransformation *in vivo*. We approached this problem by an investigation of the mutagenicities of two aromatic amines, 4-aminobiphenyl and benzidine, after bioactivation by the whole animal (HOST-MEDIATED ASSAY) and after bioactivation by a rat-liver S9 fraction (SALMONELLA/MICROSOME ASSAY). In this connection we also studied effects of bioactivation *in vitro* using intact rat hepatocytes (SALMONELLA/HEPATOCYTE ASSAY). Comparison of the various test results revealed substantial differences between the mutagenicity after metabolism *in vivo* and the mutagenicity after metabolism by a rat-liver S9 fraction. The mutagenic activity of the aromatic amines, measured after metabolism by intact rat hepatocytes, resembled the mutagenic activity after biotransformation *in vivo* more closely (chapter 3).

The utility of isolated rat hepatocytes as the metabolic factor in short-term mutagenicity assays was further evaluated by testing a number of well-known premutagenic compounds in the commonly-used Salmonella/microsome assay and in the Salmonella/hepatocyte assay. In a qualitative sense the results obtained with the two systems were on the whole equivalent. In quantitative respect, for some compounds obviously divergent mutagenic values were recorded with the different procedures (chapter 3).

Experiments *in vivo* are described in PART 2 of the thesis. Methods for the detection of mutagenic products in urine were developed and refined with the aid of animals having received precarcinogenic compounds. The chemical compounds used in this study were aromatic amines and azo dyes (benzidine-based dyes) which can be activated to be mutagenic by a series of consecutive enzymatic reactions. It became evident that N-acetylation plays an important role in the bioactivation of these compounds. Urinary metabolites were well detectable as mutagens after metabolic activation with different liver-enzyme fractions in combination with β-glucuronidase (chapter 4).

PART 3 of the thesis concerns investigations in which the applicability of the Salmonella/mutagenicity assay to detect human exposure to genotoxic

chemicals was studied. For detection of the so-called internal exposure, urine samples of persons potentially exposed to numerous chemical compounds - including unidentified mixtures - were screened for the presence of mutagens. In some cases the environment was tested for the presence of mutagenic compounds.

The study described in chapter 5 clearly shows that smoking cigarettes results in an increase in urinary mutagenicity. The presence of thioether compounds (common detoxification products of electrophilic compounds) as well as the presence of mutagens in urine were significantly related to the amount of cigarettes smoked. An increase in urinary mutagenicity was also found in urine of so-called passive smokers. This observation strongly suggests that there is a causality in the association between increased cancer risk and passive smoking, as was found by other investigators. The increase in urinary mutagenicity due to the passive exposure to cigarette smoke is about 4% of the increase observed with the active smokers during the experiment. Judging from these data it is supposed that the increased risk of cancer for passive smokers is of the same order of magnitude (chapter 5).

Many cytostatic drugs used in anti-cancer therapy were found to be genotoxic in short term assays. In accordance with this property some of them also appeared to be carcinogenic in experimental animals. For this reason these substances bring about a potential hazard to persons who are occupationally involved in the synthesis, formulation and administration of these drugs, e.g. during nursing of cancer patients. In the present study we have investigated the effect of exposure to cytostatic drugs on the mutagenicity of urine from hospital workers. A method was used in which direct effects of smoking were distinguished from effects due to exposure to cytostatic drugs. In the study of the effects of exposure to cytostatic drugs, it was found that the mutagenicity of urine from exposed smokers was significantly higher than that of the control smokers. In this respect exposed non-smokers did not differ from control smokers. These results suggest a synergistic action. On account of additional animal experiments it is supposed that this synergism is at least partly due to the induction of enzymes involved in the mutagenic activation of cytostatic drugs. On the other hand, it cannot be excluded that differences in working hygiene between smokers and non-smokers also play a role (chapter 6).

In chapter 7 a study about exposure to mutagenic substances of workers who are occupationally involved in the treatment of wood with creosote (see also chapter 1) is described. The application of a wipe-test obviously demonstrated the presence of mutagenic substances in the work environment. However, no increase in the urinary mutagenicity of creosote workers was detected in relation to their work, although administration of a relatively high dose of creosote (250 mg/kg) to rats resulted in the appearance of mutagens in urine. From this finding one must not conclude that no internal exposure has occurred during handling of the creosote.

The latter example demonstrates that in some cases a negative response in the urinary mutagenicity test can make the interpretation - in terms of the subjects' genotoxic risk - problematic. Hence, the urinary mutagenicity assay should merely be considered as a signal-test. This implies that in case of a negative result, additional tests should be performed, analogously to the use of a battery of several short-term assays in the screening for carcinogenic potency of chemicals. Whenever possible, particularly when the environmental genotoxic chemicals are known, selective (chemico-analytical) methods may be preferable. According to the present understanding it is not possible to estimate the extent of genotoxic risks on account of urinary mutagenicity values. It seems valid, however, to appraise relative risks for groups of persons being exposed under comparable conditions to similar chemical products by comparison of values of the urinary mutagenicity.

Het ontstaan van kanker door chemicaliën hangt veelal samen met het feit dat de chemische stof in kwestie genotoxische eigenschappen bezit. Dit houdt in dat de stof op de een of andere wijze een interactie kan aangaan met DNA. Deze eigenschap laat zich opsporen met behulp van zogenaamde korte-termijn testen, waarvan de Salmonella/mutageniteitstest een van de meest geëvalueerde en een van de meest betrouwbare is gebleken.

Wanneer men mensen, die mogelijkerwijs via het werk of op andere wijze veelvuldig met chemicaliën in contact komen, tracht te beschermen tegen een verhoogd risico op het krijgen van kanker, zijn twee soorten gegevens erg belangrijk. In de eerste plaats moet men kankerverwekkende eigenschappen van de betreffende chemische producten kunnen herkennen. In de tweede plaats is het van belang om in bepaalde situaties de mate van blootstelling aan kankerverwekkende stoffen te kunnen bepalen.

In dit proefschrift wordt op beide aspecten ingegaan. De Salmonella/mutageniteitstest werd gebruikt om genotoxische eigenschappen van chemicaliën vast te stellen, terwijl daarnaast mogelijkheden werden bestudeerd om in bepaalde situaties blootstelling aan mutagene c.q. potenieel carcinogene chemicaliën met behulp van deze test op te sporen.

In DEEL 1 worden enkele *in vitro* studies beschreven, waarin o.a. de Salmonella/mutageniteitstest onder verschillende condities wordt aangewend ter opsporing van mutagene eigenschappen van chemische producten.

Met behulp van de Salmonella/microsoom test werd gevonden dat creosoot P1, een tot nu toe veilig geacht houtverduurzamingsmiddel bestaande uit een mengsel van chemicaliën, duidelijk mutagene eigenschappen heeft. In tweede instantie werd deze test gebruikt om in combinatie met chemisch-analytische methoden de mutagene componenten te karakteriseren. Op deze wijze werd de aanwezigheid van benzo(a)pyreen en benz(a)anthraceen bepaald in concentraties van resp. 0,18 en 1,1% (hoofdstuk 1).

Electrofiele verbindingen of intermediairen zijn in het algemeen in staat te reageren met endogeen glutathion. Nadat was gebleken dat toediening van tolueen of xylenen aan ratten resulteerde in een afname van de concentratie van glutathion in de lever, rees de vraag of deze stoffen of hun metabolie-

ten op grond van electrofiele eigenschappen ook een mutagene werking zouden hebben. Bij het testen van deze verbindingen en van enkele metabolieten werd geen mutageen effect geconstateerd. Het feit dat een chemische verbinding electrofiele eigenschappen heeft impliceert dus niet zonder meer een mutagene activiteit (hoofdstuk 2).

Uit oogpunt van de predictieve waarde kan men zich afvragen in hoeverre de bio-activering, die in de Salmonella/microsoom test wordt uitgevoerd middels een bepaalde enzymfractie uit rattelever (S9 fractie), de biotransformatie *in vivo* weerspiegelt. Om dit te onderzoeken werd de mutageniteit bepaald van twee aromatische aminen, 4-aminobiphenyl en benzidine, zowel na bio-activering door het totale proefdier (HOST-MEDIATED TEST), als na bio-activering *in vitro* door toevoeging van een zg. S9 fractie (SALMONELLA/ MICROSOOM TEST). Ter vergelijking werd tevens het effect onderzocht van *in vitro* bio-activering met behulp van geïsoleerde rattelevercellen (SALMONELLA/ HEPATOCYT TEST). Uit dit onderzoek bleek dat er aanzienlijke verschillen kunnen bestaan tussen de mutageniteit na *in vivo* metabolisme en de mutageniteit na metabolisme door de S9 fractie. De mutagene activering met behulp van geïsoleerde levercellen benaderde het meest de biotransformatie zoals die in het totale organisme plaatsvindt (hoofdstuk 3).

Voor een verdere evaluatie van de bruikbaarheid van geïsoleerde levercellen als metabole factor bij de screening van mutagenen *in vitro* werd een aantal bekende premutagene verbindingen onderzocht op mutageniteit in de standaard Salmonella/microsoom test en in de bovengenoemde Salmonella/hepatocyt test. Over het algemeen werden er in kwalitatieve zin geen verschillen gevonden. In kwantitatieve zin werden echter voor sommige stoffen opmerkelijke verschillen geconstateerd (hoofdstuk 3).

In DEEL 2 van het proefschrift worden met name *in vivo* experimenten beschreven. Met behulp van proefdieren, die precarcinogene stoffen kregen toegediend, werden methoden voor het opsporen van mutagene producten in de urine ontwikkeld, c.q. geoptimaliseerd. De verbindingen die bij dit onderzoek werden gebruikt waren aromatische aminen en azo-kleurstoffen (zg. benzidine-based dyes), welke op verschillende wijzen kunnen worden geactiveerd volgens een ingewikkeld patroon van opeenvolgende enzymreacties. De N-acetylering bleek hierbij een belangrijke rol te spelen. Mutagene metabolieten in de urine afkomstig van dit type verbindingen konden zeer goed worden gedetec-

teerd na metabole activering, waarvoor verschillende leverenzymfracties, eventueel in combinatie met andere enzymen, werden gebruikt (hoofdstuk 4).

DEEL 3 van dit proefschrift is gewijd aan onderzoek waarin de Salmonella/mutageniteitstest werd toegepast bij het signaleren van mogelijke expositie van mensen aan genotoxische verbindingen. Het gaat in deze studies om blootstellingen aan mengsels van chemicaliën. Urinemonsters van mogelijk geëxponeerden werden onderzocht op de aanwezigheid van mutagenen als indicatie voor een "inwendige belasting". In enkele gevallen werd de omgeving getest op de aanwezigheid van mutagene stoffen.

Het onderzoek laat duidelijk zien dat roken van sigaretten resulteert in een verhoging van de mutageniteit van urine (hoofdstuk 5). Zowel de aanwezigheid van thioethers (in het algemeen ontgiftingsproducten van electrofiele verbindingen) als de aanwezigheid van mutagenen in de urine bleek gecorreleerd te zijn met het aantal gerookte sigaretten. Ook in de urine van zg. passieve rokers werd een verhoging van de mutageniteit geconstateerd. Deze waarneming ondersteunt de resultaten van epidemiologische onderzoeken, waarin een relatie werd aangetoond tussen passief roken en een vergroot risico op het krijgen van longkanker. De stijging in de mutageniteit van de urine van passieve rokers is ongeveer 4% van het verschil in mutageniteit van de urine van de actieve rokers ten opzichte van niet-rokers. Mede op basis hiervan wordt het vermoeden geuit dat het extra risico op het krijgen van longkanker voor passieve rokers in dezelfde orde van grootte ligt (hoofdstuk 5).

Het is bekend dat veel cytostatica die gebruikt worden bij de chemotherapie van kanker genotoxisch zijn en op grond daarvan ook zelf in staat blijken tumoren te induceren. Bij de behandeling van kankerpatiënten vindt, alvorens de therapie wordt toegepast, een afweging van voor- en nadelen plaats. Geheel anders is de situatie echter voor hen die beroepsmatig met deze therapeutica omgaan. Bij een onderzoek naar een mogelijke "inwendige belasting" met cytostatica van verplegend personeel werden urinemonsters onderzocht op de aanwezigheid van mutageniteit (hoofdstuk 6). Bij dit onderzoek werd een zodanige testmethode gekozen dat directe effecten van roken konden worden onderscheiden van de effecten ten gevolge van blootstelling aan cytostatica. Met deze methode werd gevonden dat verplegend personeel dat rookt een verhoogde mutageniteit van de urine had ten opzichte van controle-rokers. Een dergelijke verhoging werd niet waargenomen bij niet-rokend, verplegend per-

soneel. Deze resultaten wijzen in de richting van een synergistische werking van roken op de mutageniteit van urine bij blootstelling aan cytostatica. Op grond van dierexperimenten zou dit effect ten dele kunnen worden toegeschreven aan enzyminductie ten gevolge van het roken. Het is aannemelijk dat door een geïntensiveerde biotransformatie relatief meer van de cytostatica als mutageen in de urine verschijnt. Daarnaast kan niet worden uitgesloten dat verschillen in arbeidshygiëne tussen rokers en niet-rokers ook een rol spelen.

Hoofdstuk 7 betreft een onderzoek naar de expositie aan mutagenen van werknemers die regelmatig in aanraking komen met het genotoxische creosoot (zie hoofdstuk 1) in een houtverduurzamingsbedrijf. Door toepassing van een zogenaamde veegtest werd besmetting van de werkomgeving met mutagenen aangetoond. Hoewel een relatief hoge dosis (250 mg/kg) van creosoot toegediend aan ratten resulteerde in het verschijnen van mutagenen in de urine, werd geen verhoogde mutageniteit in de urine van werknemers gevonden in relatie tot de blootstelling tijdens het werk met het genotoxische conserveringsmiddel. Een bevinding waaruit niet geconcludeerd mag worden dat er geen "inwendige belasting" heeft plaatsgevonden.

Dit laatste voorbeeld illustreert dat een niet verhoogde mutageniteit van de urine moeilijk te interpreteren is in termen van genotoxische risico's. Een mutageniteitstest op urine dient derhalve te worden beschouwd als een signaal test. Dit impliceert dat bij een negatieve uitslag van de biologische monitoring van mutageniteit van de urine, bij blootstelling aan mengsels van chemicaliën, waaronder genotoxische producten worden vermoed, additionele testen moeten worden uitgevoerd in analogie met het gebruik van de zg. batterij van korte-termijn testen die gehanteerd wordt bij het *in vitro* mutageniteitsonderzoek. Wanneer de mogelijk belastende factoren bekend zijn dient men het gebruik van meer selectieve (chemisch-analytische) bepalingen te overwegen.

Kwantificering van het eventuele genotoxische risico op grond van de mutageniteit van urine is vooralsnog niet mogelijk. Wel kunnen in geval van vergelijkbare exposities relatieve risico's worden bepaald voor groepen van personen door de mutageniteitswaarden van urine met elkaar te vergelijken (zie bijvoorbeeld hoofdstuk 5).

Dit proefschrift was nooit tot stand gekomen zonder medewerking van vele anderen. Ik wil bij deze dan ook mijn dank betuigen aan alle medewerkers en ex-medewerkers van de Werkgroep Toxicologie en aan alle studenten die daar de afgelopen jaren een bij- of hoofdvak hebben gedaan, niet in de laatste plaats voor de prettige sfeer waaraan ieder bijdroeg. Daarnaast gaat ook mijn dank uit naar de overige leden van de Vakgroep Farmacologie. Menig medewerker van het Centraal Dierenlaboratorium ben ik dankbaar voor de goede samenwerking bij de proefdierexperimenten. Mijn dank gaat bovendien uit naar medewerkers van tekenkamer en medische bibliotheek voor de moeite die zij zich getroost hebben om aan mijn soms lastige vragen tegemoet te komen. Alle anderen die in een of andere vorm een bijdrage hebben geleverd aan de totstandkoming van dit proefschrift ben ik eveneens bijzonder erkentelijk.

De schrijver van dit proefschrift werd op 14 mei 1949 geboren te Amersfoort. Hij behaalde in 1968 het diploma HBS-B aan het St. Ludgercollege te Doetinchem. In 1970 begon hij met de studie Scheikunde aan de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen (S2) werd afgelegd in oktober 1973. Na het behalen van het doctoraal examen Scheikunde, met als hoofdvak Chemische Microbiologie, als bijvakken Organische Chemie en Dieroecologie en als uitbreiding Biochemie, trad hij in februari 1978 in dienst van de Katholieke Universiteit te Nijmegen, op grond van een subsidie van het Directoraat Generaal van de Arbeid (Ministerie van Sociale Zaken). Vanaf die datum verricht hij onderzoek binnen de Werkgroep Toxicologie van het Farmacologisch Instituut op het gebied van de arbeids- en bedrijfstoxicologie.

STELLINGEN

1. Bij de toepassing van niet-selectieve bepalingen voor 'biologische monitoring', zoals de thioethertest en de mutageniteitstest op urine, mag uit een negatieve uitkomst niet worden geconcludeerd dat geen of een te verwaarlozen blootstelling aan respectievelijk electrofiele en mutagene verbindingen heeft plaatsgevonden.

   R. van Doorn et al. (1981), Ann. Occup. Hyg. 24, 77-92.
   T. Heinonen et al. (1983), Int. Arch. Occup Environ. Health 52, 11-16.
   Dit proefschrift.

2. In de bedrijfshygiëne dient meer aandacht te worden geschonken aan de ontwikkeling van methoden voor 'environmental monitoring' van contaminaties van de werkomgeving, die aanleiding kunnen geven tot expositie via de huid. Dit geldt met name voor die gevallen waarvoor geen adequate methoden van biologische monitoring voor handen zijn.

   Dit proefschrift.

3. Het feit dat Pasquini et al. geen verschil vinden tussen de mutageniteit van urine van rokers en van niet-rokers, duidt er op dat de door hen uitgevoerde bepaling te ongevoelig is.

   R. Pasquini et al. (1982), Int. Arch. Occup. Environ. Health 50, 387-395.
   Dit proefschrift.

4. Het feit dat reeds 200 jaar geleden beroepskanker voor schoorsteenvegers werd vastgesteld, en dit risico voor de hedendaagse schoorsteenvegers nog steeds actueel is, geeft duidelijk aan, dat de kloof tussen wetenschappelijk onderzoek en vertaling van resultaten daarvan naar en door de samenleving moeilijk te overbruggen is.

   P. Pott, Cancer scroti, In: Chirurgical observations, Hawes, Clarke and Collins, London, 1775, pp. 63-68.
   C. Hogstedt et al. (1982), Scand. J. Work Environ. Health 8, suppl. 1, 72-78.
   E.S. Hansen (1983), Am. J. Epidemiol. 117, 160-164.

5. Het onderbrengen van de hydratatie van epoxides bij de fase 1 reacties van de biotransformatie is niet consequent. Analoog aan conjugaties als glucuronidering en sulfatering is additie van water te beschouwen als een synthese en dient daarom te worden gekenmerkt als een fase 2 reactie.

   J.A. Timbrell, Principles of Biochemical Toxicology, Taylor & Francis LTD, London, 1982.
   R.T. Williams, Detoxication Mechanisms, Chapman & Hall LTD, London 1959.

6. Daar het krijgen van kanker voor een belangrijk deel te wijten is aan de gevolgde leefwijze, is het van belang bij de kankerpreventie veel meer aandacht te besteden aan het geven van duidelijke voorlichting over individuele mogelijkheden om het kankerrisico te verkleinen. De 'van alles krijg je kanker' mentaliteit dient te verdwijnen.

R. Doll and R. Peto (1981), J. Natl. Cancer Inst. 66, 1192-1265.

7. Op grond van een onlangs gesuggereerd mechanisme voor de carcinogene werking van asbest, zou deze stof moeten worden gekenmerkt als een indirect genotoxisch carcinogeen en niet als een epigenetisch carcinogeen.

V.E. Archer and G.K. Livingston, In: W.N. Rom: Environmental and Occupational Medicine. Little, Brown and Company, Boston, 1983, p. 63. J.H. Weisburger and G.M. Williams,(1983), Environ. Health Perspect. 50, 233-245.

8. Het aantal colonies van gereverteerde bacteriën, dat in de conventionele Ames test als criterium voor mutageniteit wordt gebruikt, laat slechts een momentopname zien van de interactie tussen bacteriën en de testsubstantie. Toepassing van methoden waarbij een continue registratie van de bacteriëngroei plaatsvindt, verdient de voorkeur. In dit opzicht biedt de onlangs ontwikkelde 'Mutascreen' aanzienlijke voordelen.

K. Falck, Method for the Performance of a Mutagenicity Test, PCT Pat. Appl. No. PCT/FI83/0053.

9. Met de thans in gebruik zijnde methoden voor biologische monitoring van blootstelling aan mutagene en carcinogene stoffen, is het niet mogelijk uitspraken te doen ten aanzien van het uiteindelijke risico op gezondheidsschade in kwantitatieve zin.

Proceedings of the International Seminar on Methods of Monitoring Human Exposure to Carcinogenic and Mutagenic Agents, 12-15 december 1983, Espoo, Finland.

10. De bruikbaarheid van mosselen als bioaccumulatoren van milieuverontreinigende stoffen pleit niet voor de consumptie ervan.

11. Om rokers te beschermen tegen mogelijke gezondheidsschade tengevolge van het roken, gaat men over tot de productie van filtersigaretten en sigaretten met een lager teergehalte. Om 'passieve rokers' enigszins te beschermen, zou men gezien de hoge 'sidestream/mainstream' ratio voor veel toxische bestanddelen van sigaretterook, sigaretten moeten ontwikkelen die geen 'sidestream smoke' produceren.

H. Iwainsky and K. Winsel (1981), Z. Erkrank. Atm.- Org. 157, 90-102.

Nijmegen, 16 februari 1984

Rob Bos